Immunological and histological studies of the guinea pig conjunctiva.

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IMMUNOLOGICAL AND HISTOLOGICAL STUDIES

OF THE GUINEA PIG CONJUNCTIVA

BY

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ABSTRACT

1. Immediate hypersensitivity in the guinea pig conjunctiva has been investigated as a potential animal model for human allergic conjunctivitis.

2. Immediate conjunctival hypersensitivity was successfully induced to serum (rabbit, bovine, and sheep) and protein (ovalbumin, human serum albumin, horseradish peroxidase and bacterial amylase) antigens following immunization in buffered saline by intradermal or subcutaneous injection.

3. Conjunctival sensitivity to ovalbumin developed between 9 and 14 days after immunization, and was long lasting (>116 days) with or without regular topical challenge.

4. The guinea pig conjunctiva responded to topical doses of ovalbumin (0.025-2.5 mg), histamine (0.025-0.75 mg) and compound 48/80 (0.75-7.5 mg) in a dose-related fashion.

5. Responses to histamine were faster in onset (maximal at 15 minutes after challenge) compared with those to ovalbumin or compound 48/80 (maximal at 30 minutes).

6. Intradermal immunization of guinea pigs with ovalbumin (500 µg) led to high serum levels of IgG_1 antibody in blood samples taken between 14 and 56 days. Serum IgE antibody levels were low or undetectable at these times.

7. Evidence obtained using the homologous passive cutaneous anaphylaxis technique indicated the presence of two sub-classes of guinea pig IgG_1 anaphylactic antibody, possessing short (4-8 hours) and medium (2-4 days) term sensitizing passive transfer activity respectively.

8. The conjunctival response to ovalbumin was selectively inhibited in dose-related fashion by topical doses of the anti-histamine triprolidine (0.25-250 µg), the $beta_2$-adrenoreceptor agonist salbutamol (0.25-25 µg) and the anti-allergic agents disodium cromoglycate (0.25-2.5 mg) and doxantrazole (0.25-2.5 mg).

9. No significant inhibition was observed following systemic doses of the 5-hydroxytryptamine antagonists methysergide and B.W. 501C67 (both 1-10 mg/kg), the prostaglandin synthetase inhibitor indomethacin (1-10 mg/kg), or the steroidal anti-inflammatory drug dexamethasone (10 mg/kg).

10. Histological examination of the conjunctiva revealed an intense neutrophilic infiltration along the length of the bulbar and palpebral conjunctival epithelium between 4 and 16 hours after topical challenge with each of ovalbumin, histamine and compound 48/80.

11. Significantly increased numbers of eosinophils were only observed 8-48 hours after topical challenge with ovalbumin. A few eosinophils resulted from challenge with compound 48/80, whereas these cells were rare or absent following histamine challenge.

12. Immediate conjunctival hypersensitivity in the guinea pig therefore closely resembled human allergic conjunctivitis in a number of respects, and appears worthy of closer investigation as an experimental animal model for this human disease state.

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T A B L E S

GENERAL INTRODUCTION

It would be neither constructive nor particularly advantageous to embark on an extensive historical review of the progress which has taken place in the field of allergy research during the last 50-70 years. Nevertheless, some mention should be made of those more outstanding experimental and clinical observations on which our current understanding of allergy is based.

(i) Early studies in allergy

The symptoms of a seasonal condition which we now recognise as hay fever were first described by Bostock (1819) and Elliotson (1831). Preliminary evidence which indicated that the symptoms of hay fever and bronchial asthma might be connected with exposure to specific plant pollens was first put forward by Blackley in 1873. Little significance appears to have been attached to these early observations however, for why should only a small percentage of the population suffer adverse reactions to such common airborne pollens?

Magendie had reported as early as 1839 that if rabbits were repeatedly injected with serum obtained from dogs, they frequently manifested unaccountable reactions upon serum reinjection. Richet (1907), during a series of studies in which guinea pigs had been routinely inoculated with diptheria toxin and antitoxin as part of a horse antitoxin standardisation procedure, also observed the commonly fatal effects of repeated foreign serum injections. CIearly, a foreign substance which on first injection appeared harmless, became severely toxic when reinjected, provided that an interval of at least 14 days was allowed to elapse between the first and second injections.

Richet concluded from his studies that some kind of protective mechanism had been destroyed, and referred to the phenomenon as 'anaphylaxis', in contrast to the term 'prophylaxis' or favouring protection. Rosenau and Anderson (1906) were also able to show that the phenomenon of anaphylaxis described by Richet could be induced by repeated injections of a variety of foreign sera and proteins.

Auer and Lewis (1910) were the first workers to describe the symptomatic and pathological picture of guinea pig systemic anaphylaxis in greater detail. They showed that if guinea pigs were presensitized with antigen, and challenged intravenously, evidence of severe irritation became apparent within 1-2 minutes. The guinea pigs emitted spasmodic sneezes, rubbed their noses vigorously, and suffered convulsions after 5-10 minutes. Inspiration appeared to require violent effort, and the respiration rate slowed prior to ceasing completely. On post mortem examination, the lungs were grossly distended, the bronchioles constricted, and numerous haemorrhages were clearly visible on the diaphragm. Auer and Lewis further postulated that the mechanism of guinea pig systemic anaphylaxis might be connected in some way with human bronchial asthma.

Most early theories presented to explain the mechanism of the anaphylactic reaction were based on the false assumption that the causative antigen-antibody combination occurs in the bloodstream. Both Schultz (1910) and Dale (1913) were subsequently able to demonstrate that the site of the anaphylactic reaction is actually in the tissues. They placed strips of guinea pig ileal and uterine smooth muscle taken from presensitized animals in contact with the appropriate antigen solution, and observed a contraction of the ileal and uterine smooth muscle.

The pharmacological substances responsible for the anaphylactic reaction *in vivo* remained in doubt, although Dale and Laidlaw (1910) and Barger and Dale (1911) had speculated on the possible physiological role of β-imidazolylethylamine, an agent which they termed 'histamin'. It was not until 1927 that Sir Thomas Lewis observed the formation and release in antigen stimulated tissues of a mediator which he called H-substance, and which was subsequently confirmed as histamine. With its ability to increase capillary permeability, and to contract ileal and uterine smooth muscle, the important role of histamine in anaphylactic reactions was soon established beyond doubt (Best et al., 1927; Code, 1939).

(ii) The allergic diseases in man

During the fifty years which have elapsed since the early observations of Lewis, Dale, Schultz, and others, our knowledge and understanding of the causes and mechanisms which underlie an allergic reaction has increased substantially. The allergic diseases in man are now recognised to comprise a diverse range of conditions which include asthma, rhinitis, conjunctivitis, and urticaria. The most common causative antigens consist of a variety of weed and grass pollens, constituents of house dust, and animal furs or dander dust. The three important stages of the allergic response are essentially (i) the synthesis of a specific tissue bound antibody in response to primary antigen contact, (ii) secondary contact with antigen at one of the external body surfaces, and (iii) the resultant release in the tissues of a number of pharmacologically active substances which are responsible for the reaction itself.

The symptoms of bronchial asthma, which consist of wheezing, paroxysmal expiratory dyspnea, and obstructive pulmonary emphysema, are the result of bronchial muscle spasm in the upper airways, oedema of the bronchia mucosal and submucosal tissue, and excess local mucus secretion

(Dunnill, 1971). Although not universally accepted, asthma has been broadly classified as being of two basic types by Walker (1918) and Rackemann (1940). Those asthmatics who suffer an attack following exposure to a specific antigen are referred to as extrinsic, while those whose symptoms are caused by psychological or physical stimuli such as excitement or exercise are classified as intrinsic.

Allergic rhinitis and conjunctivitis, whether occurring on a seasonal (e.g. summer hay fever) or perennial basis, are characterized by a combination of excessive sneezing, nasal congestion, erhthema and oedema of the nasal and conjunctival mucosal tissues, itching of the eyes, and lachrimation.

(iii) Incidence and heredity of allergy

A number of early surveys quickly showed that between 10 and 15 per cent of the population of Europe and North America suffer from one or more allergic conditions on either a regular or occasional basis (Spain and Cooke, 1924; Pinëss and Miller, 1930; Vaughan, 1934). It also soon became apparent that a family history of allergy markedly increased the likelehood of an individual suffering from an allergic condition, although not necessarily of the same type as those occurring in close relatives. In consequence, most investigators accepted the concept of an inherited allergic tendency (Spain and Cooke, 1924; Bray, 1930; Richards and Balyeat, 1933; Ratner and Silberman, 1953), which has more recently been attributed to a specific gene (Aas, 1974).

The inheritance of an allergic trait appeared at first to obey none of the known laws of genetics (Ratner and Silberman, 1953). Work with inbred strains of mice has since shown, however, that the histocompatability linked Ir genes in this species at least may have the ability to control

the selectivity of both IgG and IgE antibody responses to specific antigens (Vaz and Levine, 1970), although they do not affect the overall ability to produce an IgE antibody response. In man, it has been similarly proposed that the tendency to develop high serum IgE levels aginst antigens such as ragweed may also be controlled by genes linked to the major histocompatability (HL-A) complex (Marsh et al., 1973). This inherited allergic tendency has futhermore been tentatively associated with a transient and genetically derived secretory IgA deficiency detectable during the first 12 months of life (Kaufman and Hobbs, 1970; Taylor et al., 1973; Soothill, 1973; Stokes, Taylor, and Turner, 1974).

(iv) <u>The nature of anaphylactic antibodies</u>

Nicolle (1907) was the first to show that anaphylactic hypersensitivity can be transferred between animals of the same species. He discovered that if the blood of a rabbit sensitive to horse serum is introduced into the bloodstream of a second non-immunized rabbit, then the recipient rabbit also becomes sensitive. The serum of guinea pigs and monkeys immunized with pollen extracts was also subsequently found to contain a humoral factor capable of transferring sensitivity (Harrison, 1934; Caulfield et al., 1937).

In man, one early demonstration of the passive transfer of local anaphylactic sensitivity was provided by Prausnitz and Kustner in 1921. They showed that the injection of a small sample of serum taken from an allergic individual (Kustner himself was sensitive to fish) into the skin of a non-allergic person rendered the area of skin in the region of the injection sensitive to the particular antigen involved.

By the early 1960's, experiments in both animals and man had therefore established that synthesis of a specific humoral factor was responsible

for conferring anaphylactic or Type I (Coombs and Gel, 1975) hypersensitivity on an individual. This serum factor, widely referred to as 'reagin', appeared capable of transferring this type of sensitivity between animals of the same species for long periods of time by becoming tissue-fixed. Although regarded by most workers as an antibody, it was not a 7S immunoglobulin, and was unable to fix complement or form an immune precipitate when tested against antigen *in vitro*.

The possibility that reaginic antibody might be a subclass of IgG or IgA antibody had been previously proposed. However, distinct differences in molecular antigenic determinants, electrophoretic mobilities, and sedimentation coefficients between reagin and IgA indicated otherwise. Reaginic antibody was clearly established as a unique antibody class by Ishizaka, Ishizaka and Hornbrook, in 1966. These authors isolated from the serum of an allergic patient an antibody activity with specific binding affinity for ragweed antigen, which lacked heavy chain antigenic determinants demonstrable for the other known immunoglobulin classes, and which possessed high activity in local passive transfer tests.

The following year, Johansson and Bennich (1967) reported an atypical multiple myeloma globulin protein present in the blood of a patient (N.D.). This protein, referred to as IgND, was subsequently found to be of the same immunoglobulin type as that described by Ishizaka et al., (1966), and both were quickly recognised as belonging to a new immunoglobulin class designated IgE (Bennich, Ishizaka, Johansson, Rowe, Stanworth, and Terry, 1968).

Reaginic antibodies possessing similar physicochemical and biological properties to human IgE have since been reported in the monkey (Ishizaka, Ishizaka, and Tada, 1969), rabbit (Zvaifler and Robinson, 1970), guinea

pig (Margni and Hajos, 1973a; Ovary, Kaplan, and Kojimi, 1976), rat (Stechschulte et al., 1970) and mouse (Provost-Danon, Binaghi, and Rochas, 1972).

In those widely used experimental animals such as the guinea pig and rat, certain IgG antibody subclasses also appear capable of conferring either local or systemic anaphylactic sensitivity (Parish, 1970c; Perini and Mota, 1973). In this connection, there has similarly been limited speculation concerning a role for IgG antibodies in human allergic reactions, particularly asthma (Parish, 1970b; Reid, 1970; Bryant, Burns and Lazarus, 1973, 1975).

(v) <u>The mechanism of the allergic reaction</u>

The close relationship which exists between immediate hypersensitivity reactions, reaginic (IgE) antibody, and tissue mast cells, has long been established. Degranulation of mast cells has been observed microscopically in response to either antigen-antibody combination (Mota, 1957, 1958; Hogberg and Uvnas, 1960), or chemical stimuli provided by substances such as compound 48/80, polymyxin, and the calcium ionophore A23187 (Riley and West, 1955; Lagunoff and Benditt, 1960; Trotter and Orr, 1974; Kagayama and Douglas, 1974; Cochrane and Douglas, 1974). Blood basophils have been shown to possess cell membrane bound IgE (Sullivan, Grimley, and Metzger, 1971; Becker et al., 1973), and to respond to the above stimuli in an essentially similar manner to mast cells (Chan and Yoffey, 1960; Shelley and Juhlin, 1962; Friedlaender and Friedlaender, 1964).

A number of authors have demonstrated positive correlations between the mast cell population and tissue content of heparin (Jorpes, 1939), histamine (Riley and West, 1953; Riley, 1955; Mota, 1958), and 5-hydroxytryptamine (Benditt et al., 1955). Both histamine and 5-hydroxytryptamine

are known to be stored prior to release in the granules of mast cells and basophils, bound to a heparin-polypeptide complex (Serafini-Francassini et al., 1969; Uvnas, Aborg, and Bergendorf, 1970).

Recently, the contributory activity of at least four additional types of mediator has been demonstrated during immediate hypersensitivity reactions in different species. Two of these, slow reacting substance of anaphylaxis or SRS-A (Kellaway and Trethewie, 1940; Brocklehurst, 1953, 1960) and eosinophil chemotactic factor of anaphylaxis or ECF-A (Kay and Austen, 1971), appear to be largely of mast cell origin. Prostaglandins of both the E and F series (Piper and Vane, 1969) and peptide kinins such as bradykinin (Brocklehurst and Lahiri, 1962; Piper, 1976) have also been detected in tissues undergoing an anaphylactic reaction.

The sequence of intracellular events which lead to the release of histamine, 5-hydroxytryptamine, SRS-A, and ECF-A, from mast cells remains poorly understood. Briefly, the process is known to be initiated by the bridging of two adjacent, mast cell bound, IgE molecule Fab regions by an antigen (Austen, 1974). This crosslinking has been postulated to effect a rearrangement of the proteins present in the mast cell membrane to form a membrane calcium channel (Fewtrell and Gomperts, 1977). It is through these 'protein functional units' or 'calcium channels' that the calcium influx known to be essential for mediator release is thought to occur (Foreman, Gomperts and Mongar, 1973). Further evidence that a calcium ion influx into the mast cell serves as the secondary stimulus for mediator release has been provided by the observation that the calcium ionophore A23187 is able to bypass the membrane calcium channels by acting as an ion carrier, thus directly stimulating histamine secretion (Foreman, Mongar, and Gomperts, 1973; Cochrane and Douglas, 1974).

Following the influx of calcium ions, intact glycolytic and oxidative

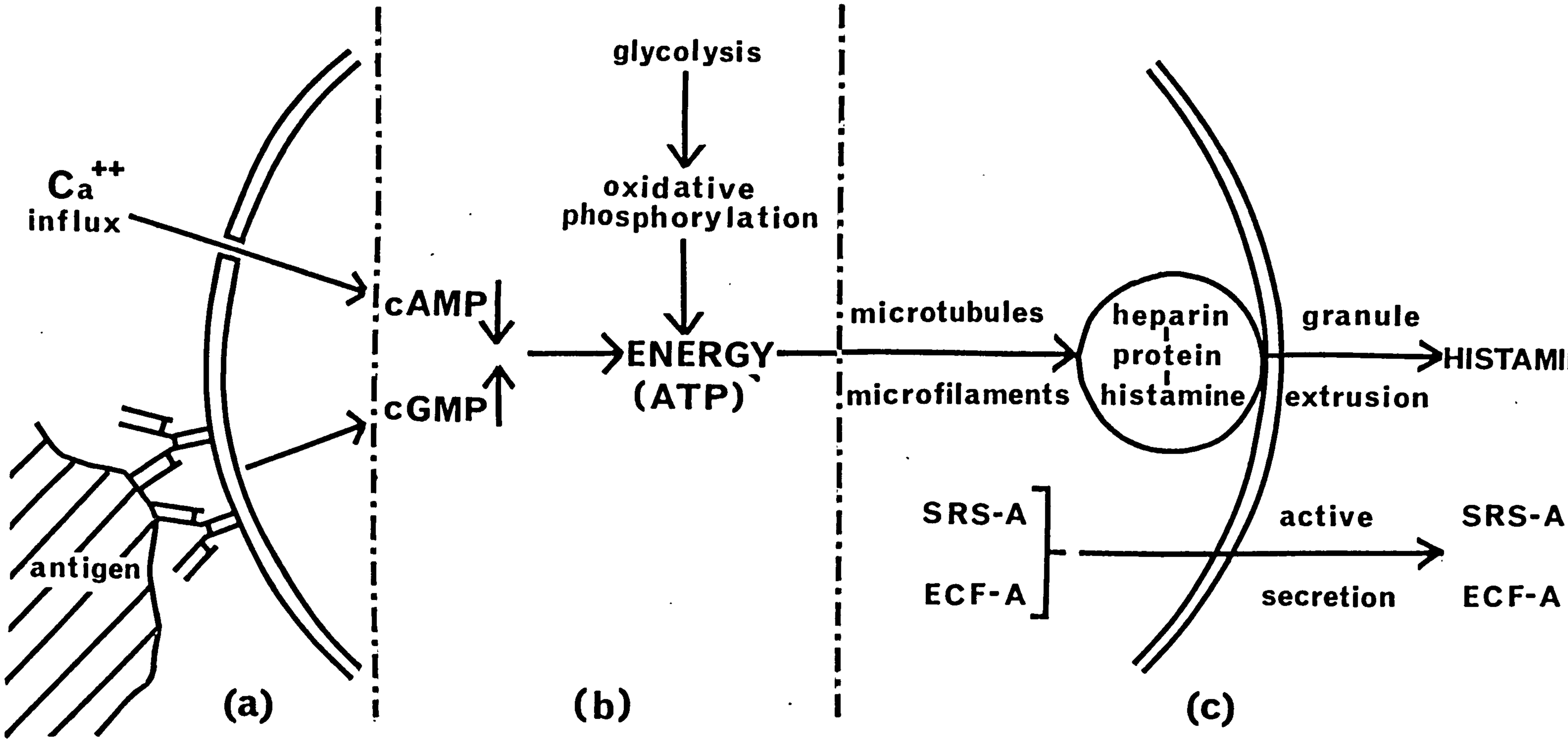


FIGURE 1. A schematic representation of the three phases of anaphylactic mediator release from mast cells:
(a) cell stimulation by crosslinking of antibody by antigen,
(b) intracellular metabolic requirement,
(c) liberation of mediators.

phosphorylation systems are required to provide energy for the release process to proceed (Orange, 1973; Austen, 1974; Peterson, 1974a, 1974b). A rearrangement of cytoplasmic microfilaments and microtubules within the mast cell is then believed to lead to the fusion of the perigranular and cell membranes, or to complete granular exocytosis (Orr, Hall, and Allison, 1972; Trotter and Orr, 1974; Tolone, Bonasera, and Parinello, 1974). Both therefore facilitate the release of histamine by simple cation exchange with sodium ions in the extracellular environment (Bergendorf and Uvnas, 1973; Anderson, Slorach, and Uvnas, 1983; Uvnas, 1974). The series of events which lead to the release of histamine, a simplified scheme for which is summarised in Figure 1, appear to be accompanied by an increase in the cyclic 3', 5'- guanosine monophosphate (cGMP) content of mast cells, and a concomitant fall in the cyclic 3', 5'- adenosine monophosphate (cAMP) level (Sullivan et al., 1975; Sullivan and Parker, 1976; Kaliner, 1977).

The release of the above anaphylactic mediators from mast cells effectively leads to the setting up of a localized inflammatory Type I hypersensitivity reaction in the tissue site. A specific cellular infiltration of easinophils and neutrophils has been demonstrated following allergic reactions in human skin (Kline, Cohen, and Rudolph, 1932), conjunctival and nasal mucosa (Loveless, 1945, Stromme, 1955), and bronchial epithelium (Morrow Brown, 1958). In the guinea pig, these cells are also characteristic of anaphylactic reactions in skin (Parish, 1972a) and conjunctiva (Dwyer, Turk, and Darougar, 1974). The mechanism and possible function of eosinophilic and neutrophilic infiltration into reactive tissues is more fully discussed in Chapter Four.

(vi) The purpose of the present study

In summary, an individual probably develops an allergy due to a combination of contributory factors. These include a possibly IgA related defect in the protection afforded by mucosal membrane barriers, a consequent increased absorption of antigen from the environment (depending on the immunogenic agent and the route and circumstances of exposure), an hereditary tendency to produce high serum levels of sensitizing antibodies following antigenic exposure, and an enhanced sensitivity to those mediators released from mast cells as a result of subsequent contact with antigen.

Clinically, the treatment of allergy has traditionally consisted of (i) the use of either skin or direct provocation tests to identify causative antigens, (ii) avoidance of contact with antigen if at all practical, (iii) courses of desensitization injections designed to induce a high blood level of IgG type 'blocking antibodies' (see Devey, Wilson and Wheller, 1976), and (iv), the use of a wide range of antihistamines, bronchodilators, and anti-inflammatory steroids in an attempt to alleviate the symptoms.

However, a more intimate understanding of asthma or hay fever will only be achieved by careful study of the diseases themselves in man, coupled with the investigation of experimentally induced related conditions in laboratory animals. A great deal of attention has therefore been focused upon the development of consistent and valid models of the human allergic state. It is from such *in vivo* (antigen induced bronchoconstriction and passive cutaneous anaphylaxis) and *in vitro* (release of mediators from rat peritoneal mast cells or guinea pig chopped lung tissue) test systems that much important information has

been obtained. These techniques have also allowed the synthesis and development of compounds possessing specific anti-allergic activity, such as disodium cromoglycate (Altounyan, 1967; Cox, 1967; Cox et al., 1970), and more recently doxantrazole (Batchelor et al., 1975), ICI 74917 (Evans et al., 1974), BRL 10833 (Spicer, Ross, and Smith, 1975), and M&B 22948 (Broughton et al., 1974).

One such animal model with a seemingly direct parallel to allergic conjunctivitis in man is the experimentally induced condition of guinea pig conjunctival anaphylaxis, first described by Feinberg and Chopra (1966). The present investigation was undertaken in order to characterize more fully the guinea pig conjunctival anaphylactic response, and to assess its suitability as a model for human allergic conjunctivitis. The results obtained are presented in Chapters I-IV, which broadly comprise:

I: A comparison of antigen immunization schedules, establishment of a conjunctival challenge technique, and the characterization and appraisal of conjunctival reaction severity.

II: Study of the serum antibody response to primary antigen immunization, and subsequent topical conjunctival challenge.

III: A description of the inhibitory activities of a number of known antagonists of immediate hypersensitivity reactions.

IV: Investigation of the cellular infiltration into the conjunctiva which occurs in response to antigen or mediator induced reactions.

CHAPTER ONE

THE DEVELOPMENT OF CONJUNCTIVAL ANAPHYLAXIS IN THE GUINEA PIG AS A MODEL OF HUMAN ALLERGIC CONJUNCTIVITIS

I N T R O D U C T I O N

An experimental 'ocular manifestation' of anaphylaxis was first observed by Ratner, Jackson, and Gruehl (1927) during a series of studies using guinea pigs sensitized either in a dust laden atmosphere, or by parenteral injection. When challenged intravenously, or by exposure to dry dander dust, many of the guinea pigs suffered severe convulsions, and died rapidly from systemic anaphylactic shock. However, a thin and watery suffusion was observed in one or both eyes of a number of survivors, comprising some 10% of the total animals tested. This conjunctival suffusion frequently became thick and creamy, and lasted for half an hour or longer after challenge. It is possible that the authors observed only a low level ocular response in those poorly sensitive guinea pigs, which had consequently survived the systemic challenge.

Almost forty years later, Feinberg, Dewdney, and Temple (1965) reported a systemic erythematous and oedematous reaction which they had observed in the skin, ears, eyelids, muzzle, and genitalia of guinea pigs during a brief period between 7 and 9 days after the intradermal injection of small amounts of foreign serum. The symptoms of this response to primary immunization with a serum antigen persisted for up to 48 hours, and were recorded in 40-75% of the guinea pigs injected. Irrespective of whether they had shown a primary sensitization reaction, all of the guinea pigs responded with an identical secondary reaction within a few hours of intradermal reinjection with the same foreign serum. They also subsequently proved to be highly sensitive to both pinnal and aerosol inhalation antigen challenge.

A later report described the immediate conjunctival and nasal reactions which had been obtained as a result of topical challenge with whole serum antigens, in guinea pigs immunized by the same method (Feinberg and Chopra, 1966). The essential symptoms of the conjunctival response comprised immediate irritation, subsequent oedema and erythema of the bulbar and palpebral conjunctiva, and a watery discharge from the eye. In addition, the severity of the conjunctival reaction appeared to be closely linked with the incidence and strength of the preceeding primary and/or secondary systemic immunization reactions.

More recently, the immediate conjunctival response to rabbit serum in the guinea pig has been more closely investigated, and compared with delayed conjunctival hypersensitivity reactions in the same species of the classic (tuberculin) and contact (dinitro-fluorobenzene) types (Dwyer and Darougar, 1971; Dwyer, Turk, and Darougar, 1974). Conjunctival anaphylaxis elicited with ovalbumin has also been employed as one of three *in vivo* tests which comprised an extensive study of the anti-allergic activity of disodium cromoglycate against allergic reactions in the guinea pig (Taylor and Roitt, 1973).

The experiments described in the first chapter of this thesis were therefore primarily directed towards the development and characterization of guinea pig conjunctival anphylaxis as an animal model for human allergic conjunctivitis. Groups of guinea pigs were immunized with either whole serum or purified protein antigens, and monitored for resultant systemic sensitization reactions. The nature and severity of the conjunctival reactions which followed topical antigen challenge were determined for each antigen sensitive group, and possible methods of response assessment were investigated. The conjunctival response to

antigen was also compared with the effect of a number of topically applied pharmacological agents. These included histamine, 5-hydroxy-tryptamine, prostaglandins of the E and F series, the mast cell mediator releasing substance compound 48/80, and the calcium ionophore A23187.

MATERIALS AND METHODS

Animals:

Outbred albino Dunkin-Hartley strain guinea pigs weighing 300-500g at immunization were used. They were obtained either as offspring from Chelsea College breeding stock (initially a gift from the Beechmans Research Laboratories, Betchworth, Surrey), or from the following suppliers:

Charles River U.K. Ltd., Margate, Kent.

Porcellus Animal Breeders Ltd.

Redfern Animal Breeders Ltd., Brenchley, Kent.

The Animal Virus Research Institute, Pirbright, Surrey.

Tuck & Sons, Rayleigh, Essex.

The guinea pigs were fed a standard pellet diet supplemented with daily greens.

Strain and species response variation studies were undertaken at Chelsea College, and at the Laboratory Animal Centre, Carshalton,Surrey (by kind permission of Dr. M. Festing), using the following animals:

Rabbits: New Zealand Whites (Chelsea stock-Buxted).

Guinea pig strains: complement (C4) deficient, R9, B, and OM3.

Rat strains: CFHB, WA, AGUS, PVGC, WAG, and LH.

Mouse strains: LACA, CBA, BALB/C, C57L, A2G, and NZB.

Serum Antigens:

Rabbit and sheep sera were supplied by the Wellcome Research Laboratories, Beckenham, Kent. Bovine serum was prepared from whole blood at Chelsea College. The sera were stored frozen at -15°C in 2ml aliquots before use.

Protein Antigens:

Ovalbumin (5x recrystallized) and human serum albumin were purchased from Koch-Light Laboratories Ltd., horseradish peroxidase from the Sigma Chemical Company, and bovine serum albumin and bovine gamma globulin from the Armour Pharmaceutical Company.

The crystalline bacterial amylase (B. subtilis) was a gift from Dr. T. Yagura at the Osaka University Medical School, Osaka, Japan.

Chemicals:

Histamine dihydrochloride and 5-hydroxytryptamine creatinine sulphate were purchased from Sigma and stored dessicated at 4°C. Batches of compound 48/80 were kindly supplied by Dr. A. F. Green at the Wellcome Research Laboratories. Prostaglandins E_1, E_2, and $F_{2\alpha}$ were purchased from Cambrian Chemicals Ltd., Croydon, Surrey, and stored in ethanol or buffer at -20°C.

Sensitization:

Guinea pigs were sensitized to the serum antigens either by a single, or two simultaneous 0.1 ml intradermal injections of serum (diluted to 20% in buffered saline and equivalent to 0.02 or 0.04 ml whole serum respectively).

Protein sensitization was by a single intradermal injection of the specified antigen dose in 0.1 ml buffered saline (pH 7.4).

Conjunctival Challenge:

Serum antigen challenge was performed by topically instilling two drops of undiluted serum onto the cornea of the eye. The eyelids were then gently opened and closed to ensure uniform distribution of antigen in the conjunctival sac.

Challenge procedure was essentially similar for protein antigens and pharmacological agents, except that an adjustable Finn pipette (Jencon) with disposable tip was used to deliver accurate topical challenge volumes in the 5-50 µl range. The standard challenge volumes were for guinea pigs: 25 µl, rats: 15 µl, and mice: 10 µl.

Intraconjunctival challenge was also used where specified, to deliver accurate antigen or mediator doses locally and directly into the sub-mucosal tissue of the upper bulbar conjunctiva. 0.02 ml of the challenge solution was injected intraconjunctivally using a 0.25 ml glass syringe and 30 gauge stainless steel short bevel needle.

Histamine and 5-hydroxytryptamine doses were calculated as base concentrations. Prostaglandin solutions were prepared by evaporating off the ethanol with nitrogen, and making up the required strength solutions in phosphate buffer (pH 7.9). The calcium ionophore was dissolved initially in ethanol or dimethyl sulphoxide, and diluted in phosphate buffer (pH 7.4).

Assessment of Conjunctival Reaction:

Four methods were investigated for accuracy and ease of assessment of reaction severity:

1. A 0 to 4 + visually scored assessment system defined as follows:

SCORE	ERYTHEMA AND OEDEMA	GROSS APPEARANCE
0	None	No symptoms detectable.
1	Slight	Reddening of the conjunctiva and eyelid margins.
2	Striking and Increasing	Both eyelids. Bulbar and palpebral conjunctiva.
3		
4	Severe and maximal	Bulbar and palpebral conjunctiva grossly swollen. Eye almost closed. Watery discharge.

2. Measurement of the width of upper bulbar and palpebral conjunctival swelling using a Vernier caliper accurate to ± 0.05 mm.

3. Measurement of the external surface temperature of the swollen conjunctiva using a thermistor temperature probe.

4. Measurement of dye extrusion into the conjunctiva during reaction using the Diffusion Systems Ltd. Mark III reflectometer shown in Figure 2. In principle, light from the 6v lamp is directed through an adjustable optical filter and the apperture of an annular photocell onto the test surface. Light reflected from the test surface causes an output from the annular photocell which is measured on a galvanometer in millivolts.

   Each guinea pig was given a 20 mg/kg intravenous injection of Evan's Blue Dye (G. T. Gurr Ltd.) immediately before topical or intraconjunctival challenge. With the red filter in position, the reflectometer was placed over the control (unchallenged) and then test (challenged) eyes of each guinea pig 30 minutes after challenge. The reduction in reflectance of the challenged eye over the control was therefore determined.

Blood Smear Counts:

White cell counts were performed on air dried and alcohol fixed guinea pig blood smears stained with Giemsa (R. A. Lamb Ltd.). On each slide, 200-300 cells were counted in random microscope fields, and the results were expressed as percentage differential counts.

Precipitin Testing:

Sera from antigen sensitive guinea pigs were tested for the presence of specific precipitating antibody by immunodiffusion in New Zealand

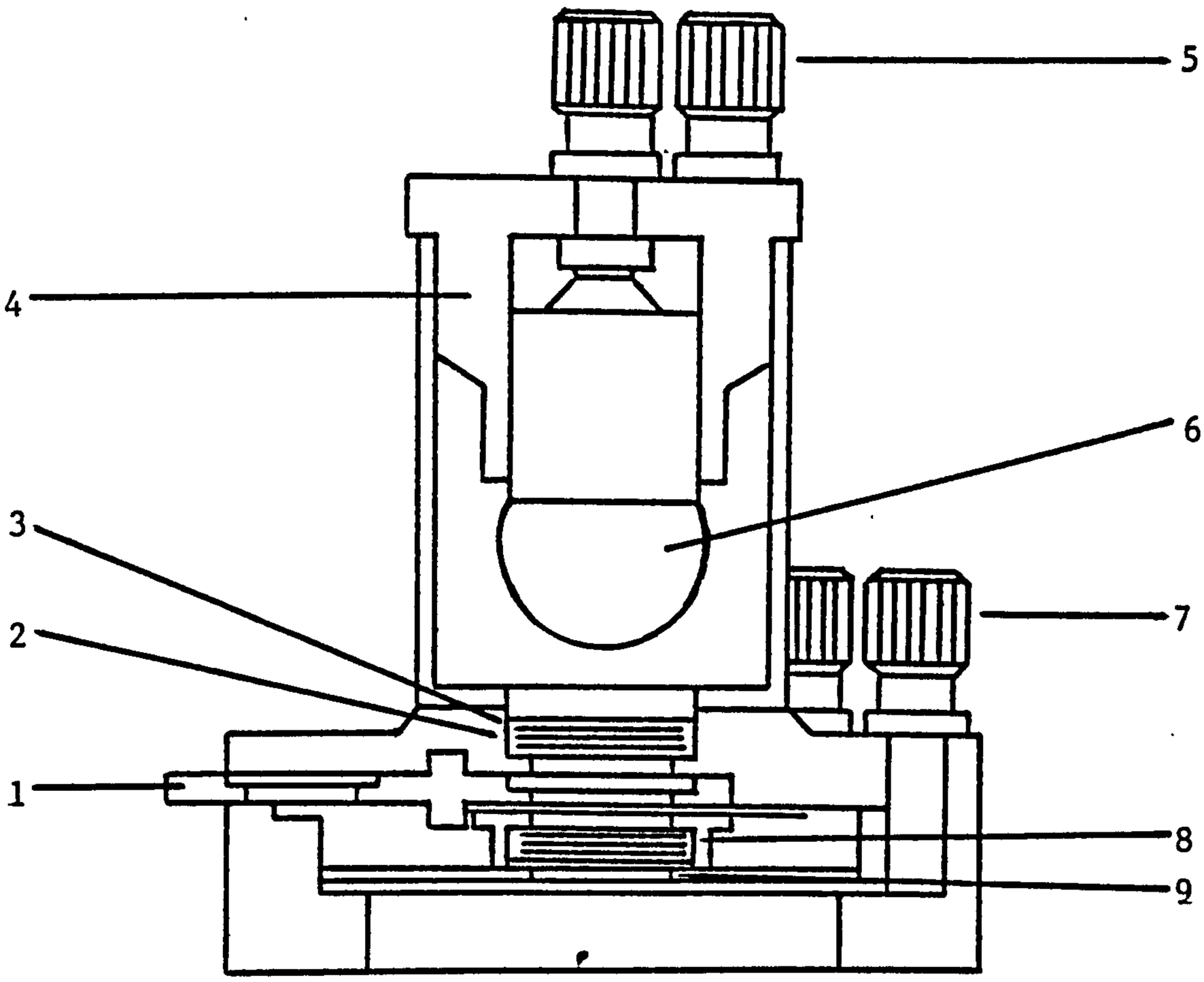


FIGURE 2. THE DSL MARK III REFLECTOMETER: MODEL 11.

GENERAL LAYOUT

(SCALE: X 1·5)

KEY:

1. FILTER WHEEL.
2. HEAT SHIELD.
3. LENS.
4. BULB HOUSING.
5. BULB TERMINALS.
6. LIGHT SOURCE : 6v BULB.
7. PHOTOCELL TERMINALS.
8. LENS/DIFFUSING GLASS.
9. PHOTOCELL.

agar gel plates (Feinberg, 1957). The plates were set up and left for 48 hours at 4°C, stained with Coomassie Blue, and preserved as described by Ouchterlony (1967).

Gel Filtration:

A 2.0 ml sample of rabbit serum was chromatographed on a 2.5 x 100 cm Sephadex G-200 gel filtration column, and eluted with phosphate buffer pH 7.4. The protein content of the eluate was monitored by passage through an LKB UVICORD 4701 A Ultraviolet Absorptiometer connected to a pen recorder (George Washington Oscillograph Type 400 MD 2). Fractions were collected on an LKB 7000 A Ultrorac fraction collector, pooled, and reduced to the original column application volume by dialysis in visking tubing against carbowax.

Statistics:

Mean conjunctival reaction scores and standard errors were calculated for most experimental treatment groups. The Wilcoxon Rank Sum Test (see Materials and Methods section, chapter Three of this thesis) was used to compare treatment groups where stated, with $p < 0.05$ being taken as indicating a significant result.

R E S U L T S

1.1. Systemic Sensitization Reactions Following Immunization with Serum Antigens.

Guinea pigs were immunized with three serum antigens: rabbit, bovine, and sheep, and observed for systemic sensitization reactions. Primary reaction symptoms were seen on the 8th day in 7 of 15 rabbit serum immunized guinea pigs, only 2 of 15 bovine serum injected guinea pigs, and none of the sheep serum immunized group (Table 1). Briefly, the reactions were characterized by extensive erythema of the ears, eyelids, feet, and skin, together with localized tissue oedema in the same areas (notably the ears). In most cases, the symptoms persisted for approximately 24 hours before subsiding.

Booster intradermal injections (equivalent to 0.02 ml of serum and given not less than 14 days later), produced strong systemic reactions within 2-3 hours in all 15 of the rabbit serum group, and 10 of the 15 bovine serum group. Once again, no systemic reactions were seen in any of the sheep serum immunized group.

Differential white blood cell counts were performed on a series of blood smears taken before, during, and after the primary sensitization symptoms shown by guinea pigs from the rabbit serum group (Table 2). Fourfold increases in blood eosinophil levels were recorded on the day of appearance of systemic symptoms (day S), and also on the following day (S+I). A 6-11 fold increase in the basophil count was noted on the next two days (S+II and S+III). No significant changes were seen in the lymphocyte or neutrophil counts on any of these four days.

Table 1. Guinea pig sensitivity to serum antigens.

Serum antigen:	Positive Response (%)		
	Rabbit (n=15)	Bovine (n=15)	Sheep (n=6)
Primary systemic reactions:	47	13	0
1st conjunctival challenge:	47	93	83*
Secondary systemic reaction:	100	66	0
2nd conjunctival challenge:	75	87	83*

Charles River outbred guinea pigs were immunized with serum antigens as described in the text. Each guinea pig was observed for the onset of either primary or secondary systemic immunization symptoms as appropriate, and for conjunctival sensitivity to topical challenge. Results are expressed as % positive responders for each treatment.

* weak responses in all cases.

Table 2. White blood cell counts (%) prior to, during, and following primary systemic sensitization reactions to rabbit serum.

Day	Lymphocytes	Eosinophils	Basophils	Neutrophils	Monocytes
S-I	40.1	1.9	0.2	56.1	1.7
S	36.2	4.9	0	57.2	1.7
S+I	32.6	7.8	1.3	55.7	2.6
S+II	38.2	3.4	2.2	54.4	1.8
S+III	39.9	2.4	2.0	53.5	2.2

Differential blood cell counts were performed on smears of blood taken from 7 guinea pigs on days prior to (S-I), during (S), and following (S+I, S+II, S+III) primary systemic sensitization reactions. The results are expressed as percent differential counts of each cell type present.

1.2. Topical Conjunctival Challenge with Serum Antigens.

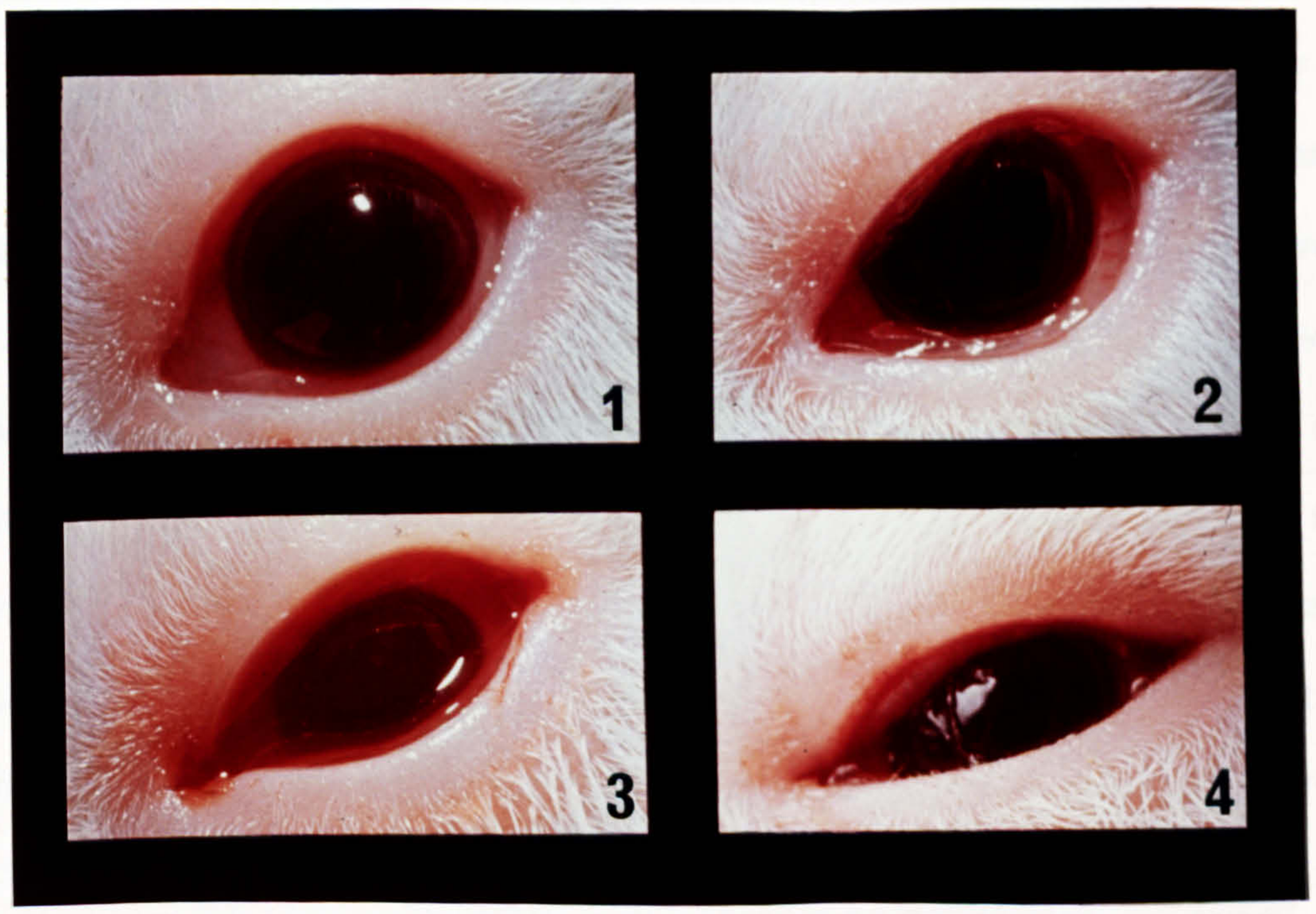
Not less than 9 days after observation of either primary or secondary systemic sensitization symptoms in reacting animals, all guinea pigs, whether systemic responders or not, were topically challenged in one eye by the instillation of two drops of the appropriate serum antigen. Immediate signs of irritation such as scratching or blinking were frequently observed in positively responding animals. Signs of erythema appeared along the margins of the eyelids within 2-3 minutes, and spread to the bulbar and palpebral conjunctiva. Conjunctival oedema developed over the period 5-15 minutes after challenge. The overall severity of the reaction was generally maximal between 20 and 30 minutes after challenge. A watery discharge from the eye was also frequently observed.

Following primary immunization, 7 of 15 rabbit serum and 14 of 15 bovine serum injected guinea pigs gave a positive conjunctival response. After secondary accelerated (booster) systemic reactions, a positive conjunctival response was recorded in 9 of 12 rabbit serum and 13 of 15 bovine serum immunized animals. Sensitivity to conjunctival challenge was observed in 5 of 6 sheep serum immunized guinea pigs tested, but the degree of sensitivity was marginal.

No symptoms of conjunctival reaction were seen in the contralateral control eye (unchallenged or saline challenged) of any guinea pig tested. Similarly, no reactions occurred in the eyes of unsensitized guinea pigs topically challenged with serum antigens.

Conjunctival reactions in response to serum antigens were assessed on the basis of a visual 0 to 4 + scale as defined in Materials and Methods, and shown in Figure 3.

(a)



(b)

Figure 3. The guinea pig conjunctival response to antigen at 30 minutes after topical challenge: (a) antigen and saline challenged eyes on a single animal, and (b) the stages of reaction severity (1-4 +) as defined in the visual assessment system. Note the intense erythema and oedema evident throughout the bulbar and palpebral conjunctival tissue.

1.3. The Nature of Serum Antigens.

Immunodiffusion agar gel plates were set up to test sera from guinea pigs immunized with both rabbit and bovine serum for specific precipitating antibody activity against these antigens. After incubation for 48 hours at 4°C, multiple precipitation lines had formed between the antiserum and antigen wells, as shown in Figure 4. Precipitating antibody activity was, therefore, clearly present against a number of serum antigen components in each guinea pig antiserum tested. Two experiments were subsequently performed to provide additional information about which foreign serum components might be antigenically active in the immediate conjunctival hypersensitivity reaction.

Firstly, six actively sensitive guinea pigs were topically challenged in the left eye with bovine serum. The reactions were scored and the animals allowed two hours for recovery. The same guinea pigs were then challenged in the contralateral (right) eye with two drops of bovine serum albumin (35 mg/ml), followed one hour later by two drops of bovine gamma globulin (20 mg/ml). None of the six guinea pigs tested showed any conjunctival response to either bovine serum albumin or gamma globulin. Finally, whole serum challenge in the right eye produced strong conjunctival reactions comparable to those observed in the left eye some 4-5 hours earlier.

Secondly, a sample of rabbit serum was fractionated on a 2.5 x 100 cm Sephadex column. Eluate from the column was monitored for protein content by absorption at 280 nm. Fractions from each of the resultant three main peaks were collected, pooled, and reduced to the original sample column application volume (2 ml). The pools from peaks I, II, and III were assumed to contain principally macroproteins, gamma globulins,

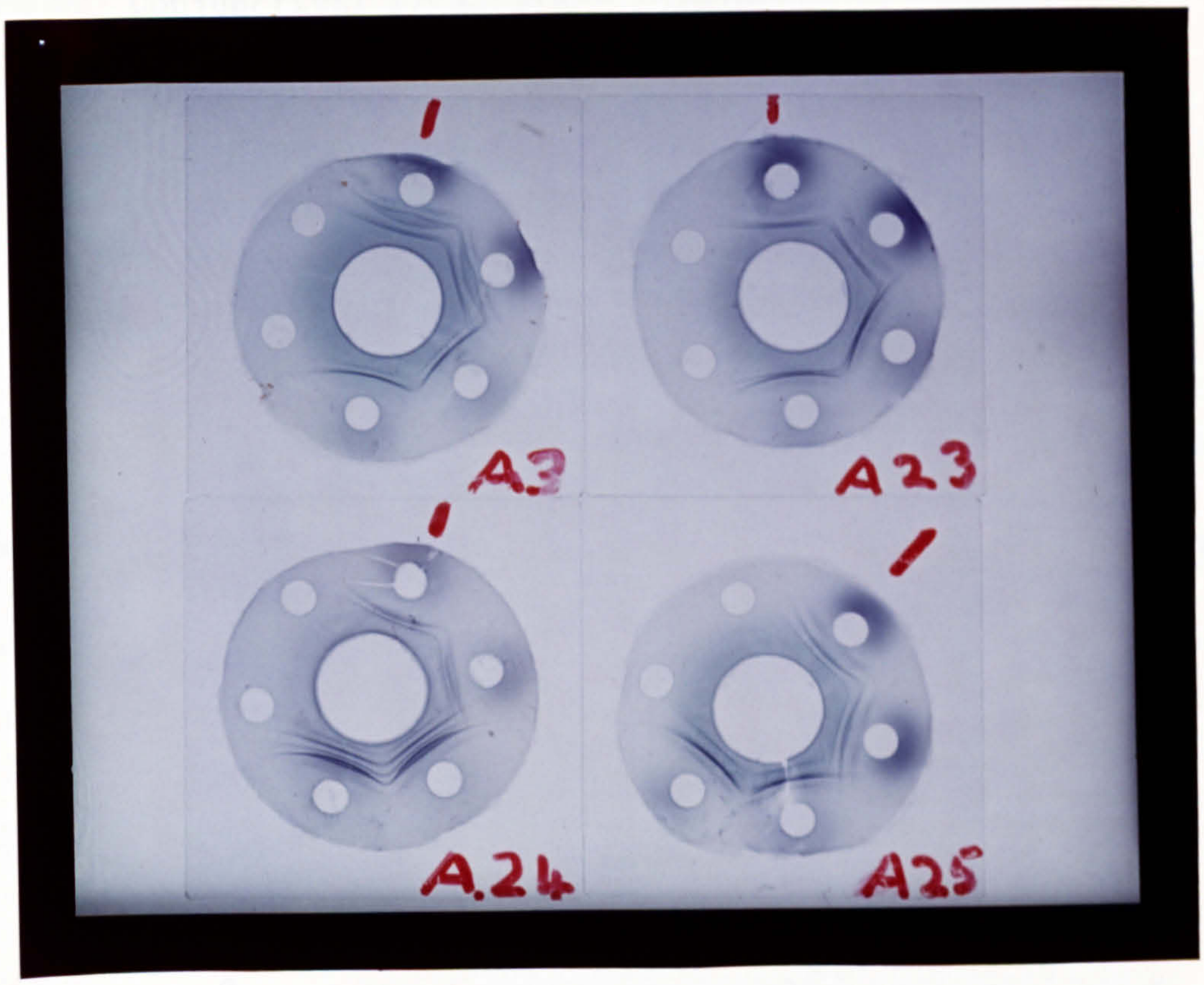


Figure 4. Agar gel immunodiffusion plates set up to demonstrate the presence of precipitating antibodies against serum antigens in conjunctivally sensitive guinea pigs. The antisera (centre wells) were obtained from four guinea pigs immunized with both rabbit serum (placed in outer wells 1 and 2 clockwise from the red marker) and bovine serum (outer wells 3 and 4). Outer wells 5 and 6 contained saline. Multiple precipitation lines were observed between all four guinea pig antisera and both serum antigens after incubation of the plates at 4°C for 48 hours, thus confirming precipitating antibody activity against multiple serum proteins. The plates were stained with Coomassie Blue, preserved, and mounted, according to Ouchterlony (1967).

Table 3. Conjunctival sensitivity to pools I, II and III of Sephadex G-200 fractionated rabbit serum.

	Left eye	Left eye	Left eye	Right eye
Animal	Pool I	Pool II	Pool III	Whole rabbit serum
1	0	+ +	+ + +	n.d.*
2	0	0	0	+
3	0	0	0	+ +
4	0	±	±	+ +
5	0	0	0	+ +

A sample of rabbit serum was chromatographed on a 2.5 x 100 cm Sephadex G-200 column. Eluate was pooled from the three main absorption peaks (see text), reduced to column application volume, and used to challenge guinea pigs sensitive to whole rabbit serum.

n.d. : not done.

* both eyes used to test pools II and III. This animal had given a 4 + response to whole rabbit serum 7 days earlier.

and serum albumin respectively, as described for human serum by Flodin and Killander (1962). Five guinea pigs sensitive to rabbit serum were then topically challenged with two drops of each of pools I, II, and III. Only two of the five guinea pigs reacted to any of the three fraction pools (Table 3). The first animal reacted strongly to both pools II and III. All five guinea pigs responded with normal strength reactions when tested with whole rabbit serum.

1.4. Topical Conjunctival Challenge with Protein Antigens.

Guinea pigs were immunized with four protein antigens; ovalbumin, human serum albumin, bacterial amylase, and horseradish peroxidase; either by intradermal or subcutaneous injection. No 8th day primary sensitization systemic reactions were observed with any of these protein antigens.

Conjunctival challenge was performed 14-21 days after immunization by the instillation of 25 µl of protein solution on to the corneum of the eye. The challenge dose was 500 µg in all cases. The reactions which resulted from conjunctival challenge with protein antigens were identical in rate of onset and appearance to those previously described for serum antigens (Figure 3).

Conjunctival sensitivity was subsequently routinely induced to ovalbumin, and on other occasions to horseradish peroxidase, human serum albumin, and crystalline bacterial amylase. Ovalbumin immunized guinea pigs showed strong conjunctival sensitivity during preliminary experiments, and continued to do so throughout the project. The results of the preliminary experiments with protein antigens are summarised in Table 4.

Table 4. Conjunctival sensitivity to protein antigens.

	Immunization			Conjunctival sensitivity	
Antigen	Dose	Route	n	% response	Reaction mean ± s.e.m.
OVA	250 µg	i.d.	11	100	2.6 ± 0.2
OVA	500 µg	i.d.	10	100	1.9 ± 0.2
OVA	500 µg	s.c.	10	90	2.0 ± 0.3
HSA	500 µg	i.d.	3	100	2.0 ± 0.6
BA	500 µg	i.d.	7	100	2.2 ± 0.3
HP	1.0 mg	i.d	7	0	0
HP (boost)	1.0 mg	i.p.	7	43	1.0 ± 0.3

Guinea pigs were immunized with ovalbumin (OVA), human serum albumin (HSA), bacterial amylase extracted from B. subtilis (BA), and horseradish peroxidase (HP) by intradermal (i.d.), subcutaneous (s.c.), or intraperitoneal (i.p.) injection at the doses stated. All were topically challenged not less than 14 days after immunization. Conjunctival reactions were visually assessed at 30 minutes, and results expressed as group mean scores ± standard error of the mean (s.e.m.).

1.5. The Effect of Conjunctival Challenge with Anaphylactic Mediators and Mast Cell Mediator Releasing Agents.

Guinea pigs were challenged topically and by intraconjunctival injection with a series of doses of histamine, 5-hydroxytryptamine, prostaglandins E_1, E_2, and $F_{2\alpha}$, compound 48/80, and the calcium ionophore A23187.

Slight irritation and minor reddening of the eyelid margins was observed in response to topical challenge with low doses of histamine (2.5 and 25 µg). Higher doses (250 and 750 µg) resulted in the extensive conjunctival oedema and erythema characteristic of antigen induced reaction. Intraconjunctival challenge with histamine elicited discrete reactions in the upper bulbar conjunctiva at low doses (1-10 µg), and severe whole conjunctiva reactions at high doses (30-100 µg).

At similar topical dose levels to those used for histamine, 5-hydroxytryptamine caused no observable oedema or erythema. A ten fold increase in dose (2.5 mg) produced weak (±) responses, as did intraconjunctival doses in the 100-300 µg range.

The three prostaglandins were tested at topical doses up to 25 µg, and intraconjunctival doses over the range 1-10 µg. No conjunctival oedema or erythema was observed with any of the three prostaglandins at any of the doses tested. Some individual guinea pigs showed evidence of local irritation (scratching, watering of the eye) at the highest doses of prostaglandin E_1.

At topical doses of 500 µg and 1.25 mg, compound 48/80 also produced evidence of irritation including blinking, lachrymation, and scratching, but no oedema. Following increased doses (2.5 and 5.0 mg) these signs

were observed prior to the development of conjunctival oedema and erythema as previously described. Intraconjunctival doses of compound 48/80 at 0.1 and 1.0 mg levels also produced clear reactions within 5-10 minutes of challenge.

The calcium ionophore A23187 elicited conjunctival reactions only at comparatively high topical (250 µg-1.0 mg) and intraconjunctival (30-100 µg) doses.

1.6. Dose-Response Relationship of the Conjunctival Reactions.

Dose-response data was obtained in guinea pigs topically challenged with a range of doses of ovalbumin, histamine, or compound 48/80. Challenge and assessment were both performed blind, and reactions were visually scored 30 minutes after challenge. Resultant typical raw data dose-response curves are shown for ovalbumin (Figure 5), histamine (Figure 6), and compound 48/80 (Figure 7). All three types of conjunctival reaction proved to be highly dose dependant.

Ovalbumin dose-response curves were of similar slope, but showed slight variation according to guinea pig batch sensitivity. Topical ovalbumin doses between 250 µg and 2.5 mg regularly produced severe conjunctival oedema and erythema.

Conjunctival dose-response curves to topical histamine challenge remained remarkably consistent in all experiments (Figure 6). Severe reactions (3-4 +) were always recorded with 250 and 750 µg doses. High doses of compound 48/80 were required to elicit strong (2 +) conjunctival oedema and erythema. The three separate batches of compound 48/80 tested (K4023, K7755, and K9308) showed little variation in activity (Figure 7).

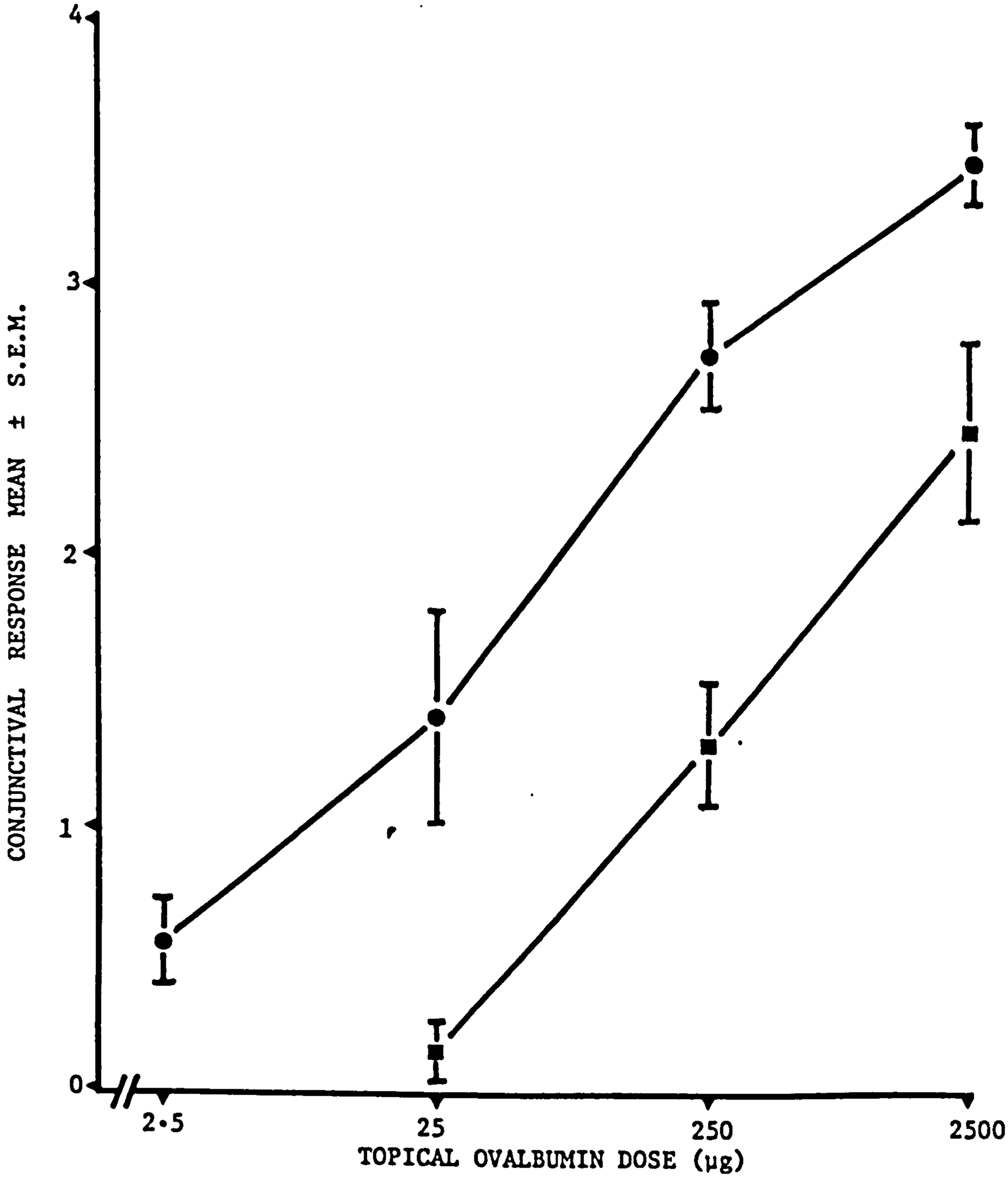


FIGURE 5. The dose-related conjunctival response to topical ovalbumin challenge.

Guinea pigs were immunized with ovalbumin (500 µg) by intradermal injection, and topically challenged not less than 14 days later. The conjunctival reactions were visually assessed at 30 minutes after challenge. Results are shown for two batches of guinea pigs with 9 (●-●) and 8 (■-■) animals per treatment group. In the latter case, a lower degree of sensitivity is demonstrated by a lateral shift in the dose-response curve to the right.

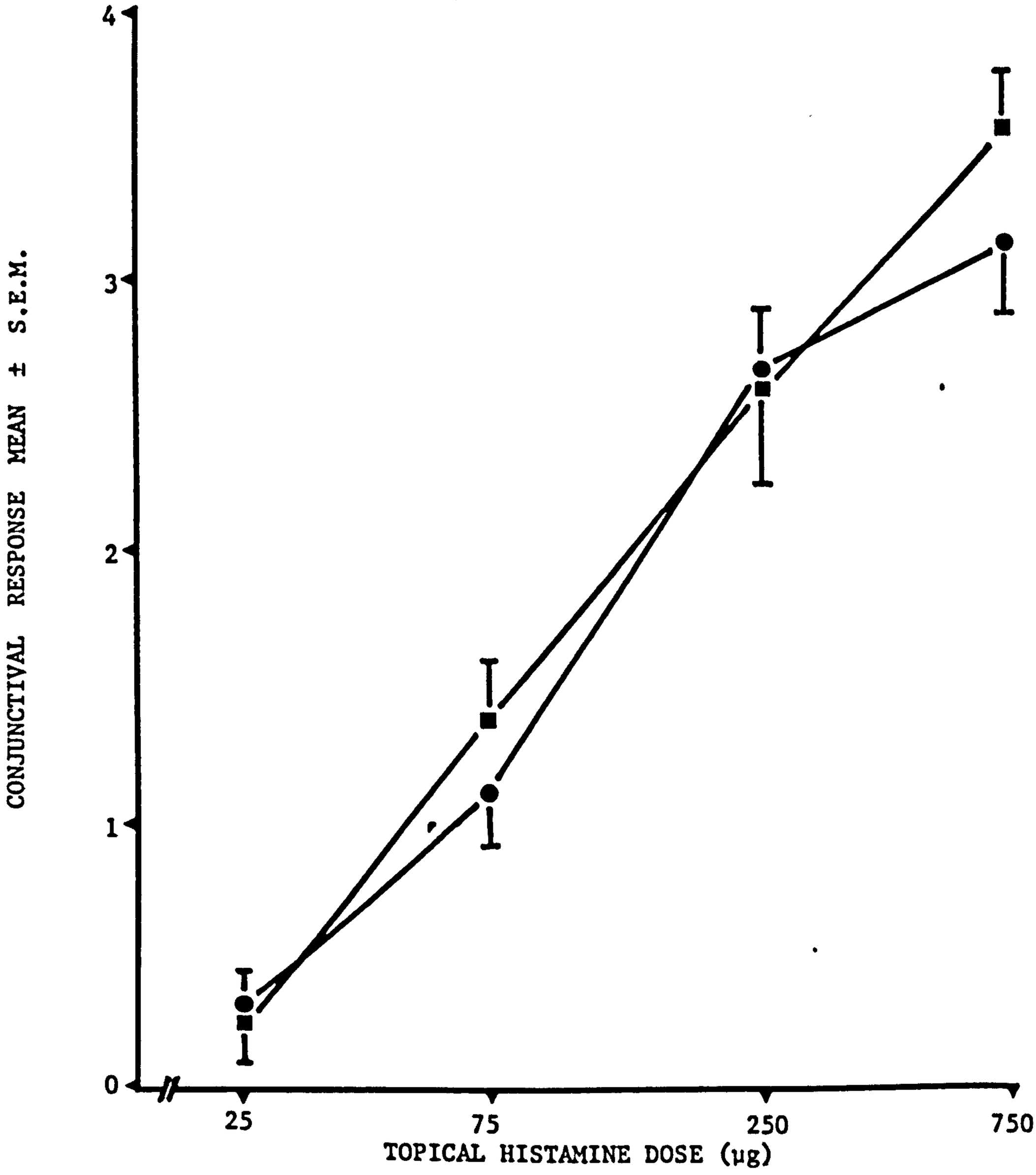


FIGURE 6. The dose-related conjunctival response to topical histamine challenge.

The results shown are taken from two separate batches of guinea pigs with 15 (● - ●) and 8 (■ - ■) animals per treatment group. Conjunctival reactions were visually assessed at 30 minutes after challenge. There was no statistically significant difference between the data obtained on each occasion.

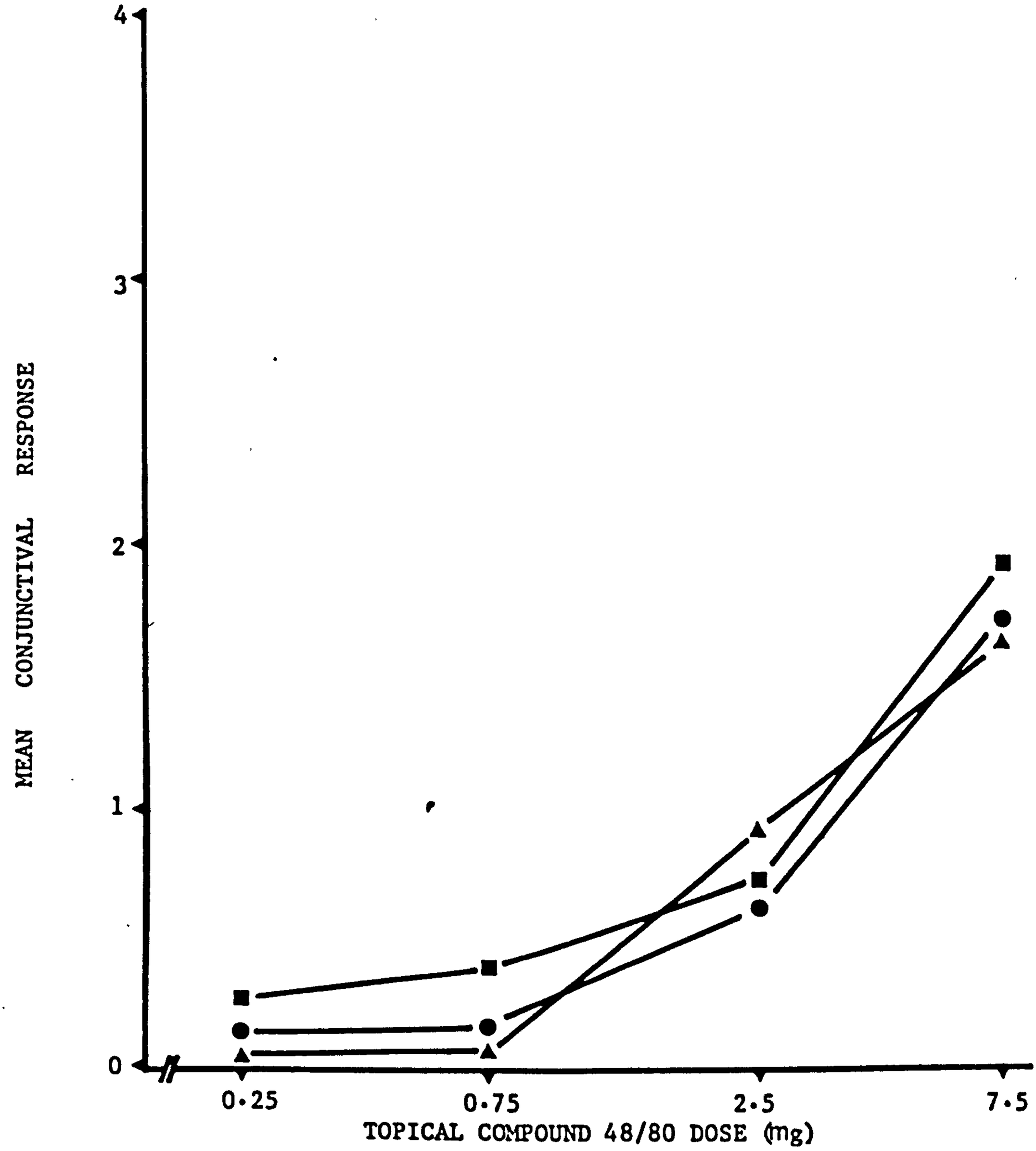


FIGURE 7. The dose-related conjunctival response to topical compound 48/80 challenge.

Three samples of compound 48/80 numbered K4023 (●-●), K7755 (■-■) and K9308 (▲-▲) were tested, using 15, 15 and 10 guinea pigs per treatment group respectively. The reactions were visually assessed at 30 minutes after challenge. There was no statistically significant difference between the data obtained on each occasion.

Dose-response curves were also determined for intraconjunctival challenge with ovalbumin, histamine, compound 48/80, and 5-hydroxytryptamine. The results largely confirmed the information obtained with topical challenge. Both ovalbumin and histamine gave positive conjunctival responses with injection doses as low as 1-2 µg (Figure 8). Dose-related weak responses were observed with 5-hydroxytryptamine over the 30-300 µg range. Histamine therefore proved approximately 300 times as active as 5-hydroxytryptamine in inducing local erythema and oedema in the guinea pig conjunctiva.

The possible effect of challenge volume on the conjunctival response was also investigated. A range of four challenge volumes (5, 10, 25 and 50 microlitres) were tested with set doses of ovalbumin (500 µg), histamine (250 µg) and compound 48/80 (7.5 mg). The responses at different challenge volumes showed little variation for ovalbumin (1.2 ± 0.3 - 1.7 ± 0.3), histamine (1.9 ± 0.2 - 2.4 ± 0.2) or compound 48/80 (0.8 ± 0.2 - 1.3 ± 0.2). No significant difference was observed between any challenge volume for any of the three reagents. In accordance with these results, 25 microlitres was routinely used as the standard volume for topical challenge in all studies.

1.7. Time Courses of the Different Conjunctival Reactions.

Three groups of ten guinea pigs were topically challenged with ovalbumin, histamine, and compound 48/80 (500 µg, 250 µg, and 7.5 mg doses respectively). They were scored for severity of conjunctival reaction by visual assessment at 5, 10, 15 and 30 minutes after challenge, and at 30 minute intervals thereafter. The reaction time courses for the three types of challenge are shown in Figure 9.

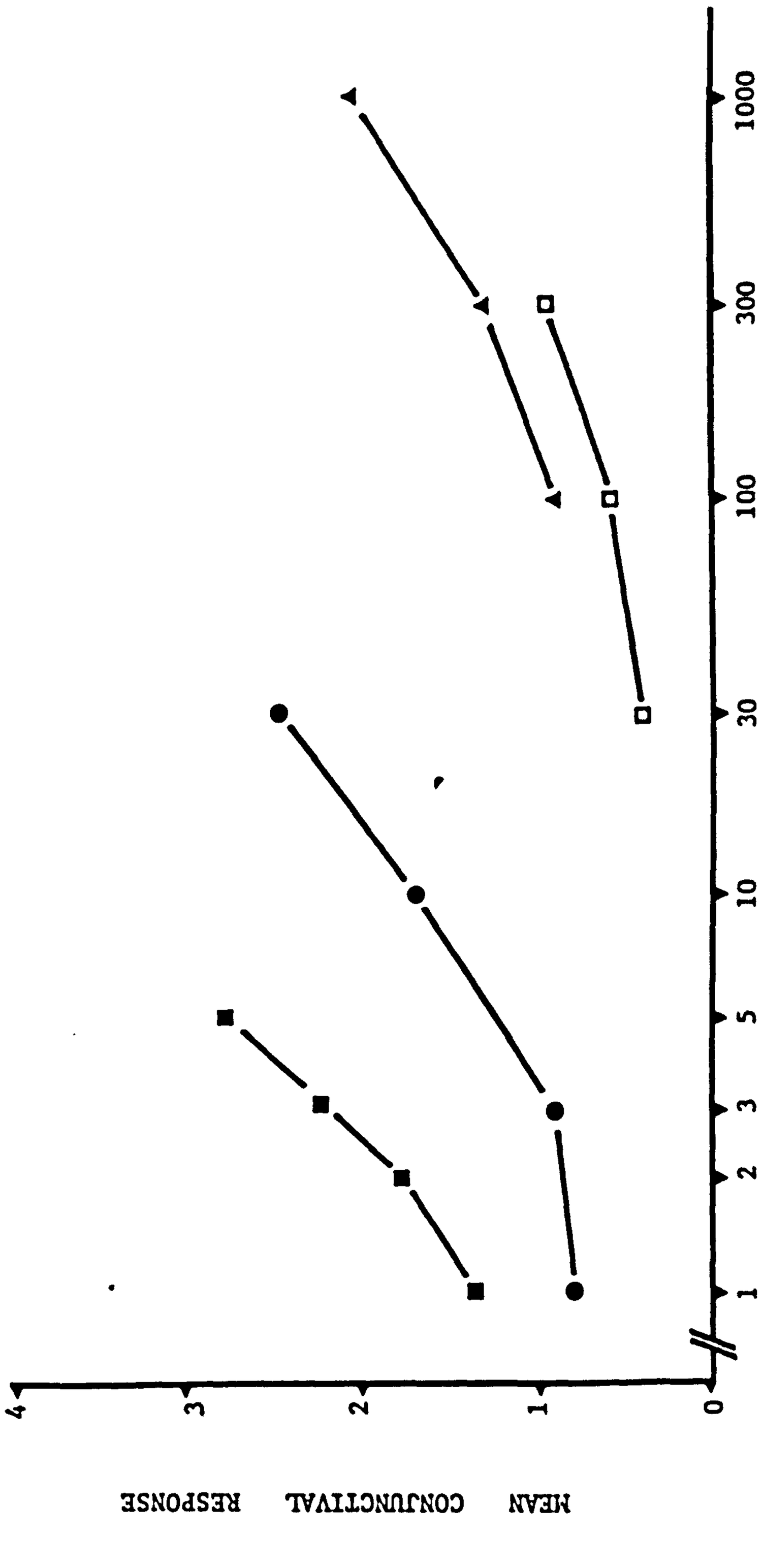


FIGURE 8. The dose-related response to intraconjunctival injection of ovalbumin (●-●), histamine (■-■), 5-hydroxytryptamine (□-□) and compound 48/80 (▲-▲).

Intraconjunctival injection doses were administered as described in the text in a volume of 0·02 ml. Reactions were visually assessed at 30 minutes after challenge. Treatment group sizes were not less than 8 guinea pigs per dose level in each case.

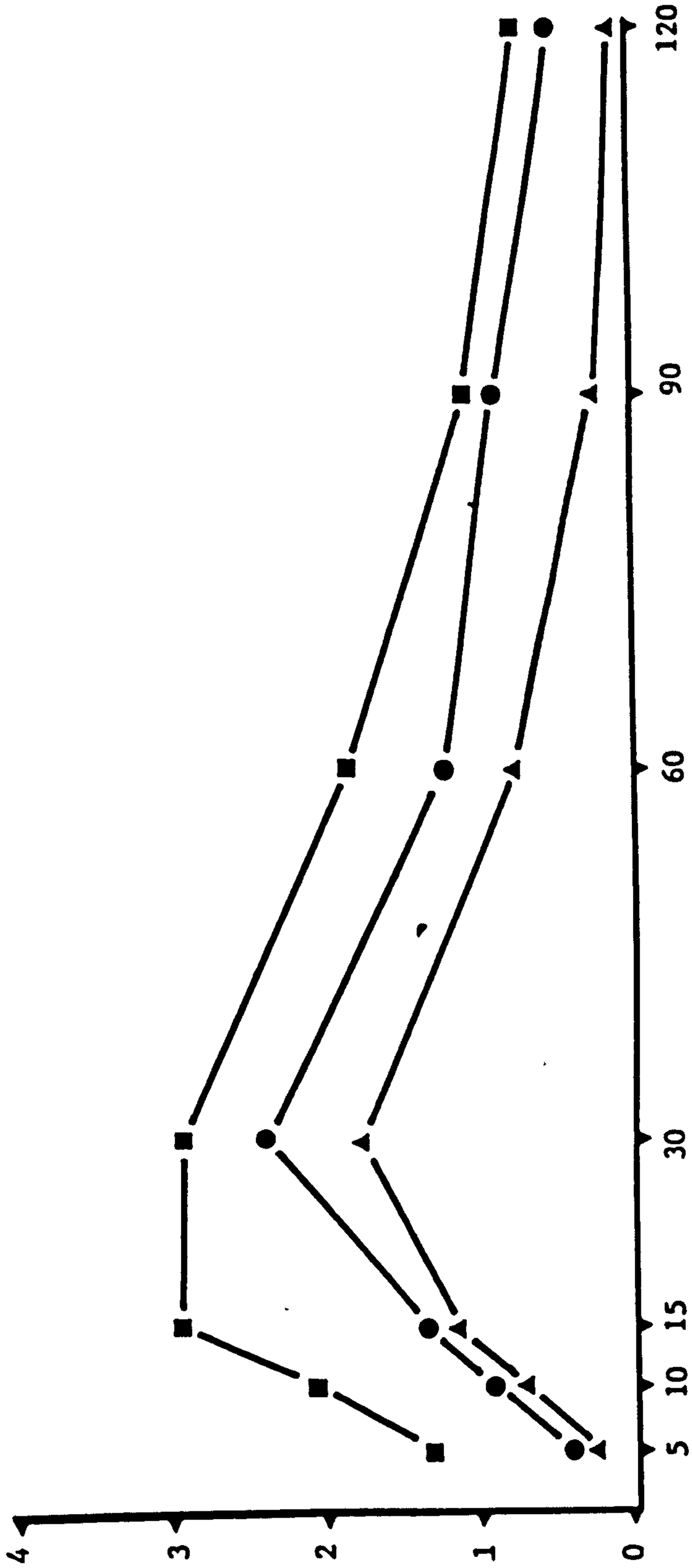


FIGURE9. Conjunctival response time courses for ovalbumin (250 µg : n = 10), histamine (■-■) and compound 48/80 (▲-▲) topical challenge.

Guinea pigs were topically challenged with either ovalbumin (500 µg : n = 10), histamine (7·5 mg : n = 8). Visually assessed group mean conjunctival responses at each of seven time intervals after challenge.

The most striking observation was the speed of onset of the histamine response. Irritation was immediately evident, and the margins of the eyelids began reddening within 2-3 minutes of challenge. Erythema and oedema of the bulbar and palpebral conjunctiva appeared after 3-5 minutes, were maximal by 15 minutes, and unchanged at 30 minutes. Antigen (ovalbumin) and compound 48/80 reactions showed a similar but rather slower onset of symptoms, and reached their peak at 30 minutes after challenge.

The recovery curves for all three types of reaction were essentially parallel in nature. The symptoms had usually receded and the eyes were normal in appearance between 120-180 minutes after challenge. Severe (4+) reactions frequently required up to 4 hours for complete recovery.

1.8. Onset and Duration of Antigen Sensitivity.

Three groups of guinea pigs received standard 500 µg doses of ovalbumin in 0.1 ml phosphate buffered saline given by intradermal (groups I and II) or subcutaneous (group III) injection. The onset of detectable conjunctival sensitivity was studied by topically challenging selected animals from each group over the period 8-14 days after immunization. All three groups were then left for a further 14-21 days and monitored for sensitivity to regular antigen challenge during the subsequent 5-13 weeks.

The first detectable conjunctival sensitivity was recorded in guinea pigs from all three groups at 9 days after immunization. Sensitivity increased in those animals tested on days 10 and 11, and was maximal by the 14th day (Figure 10).

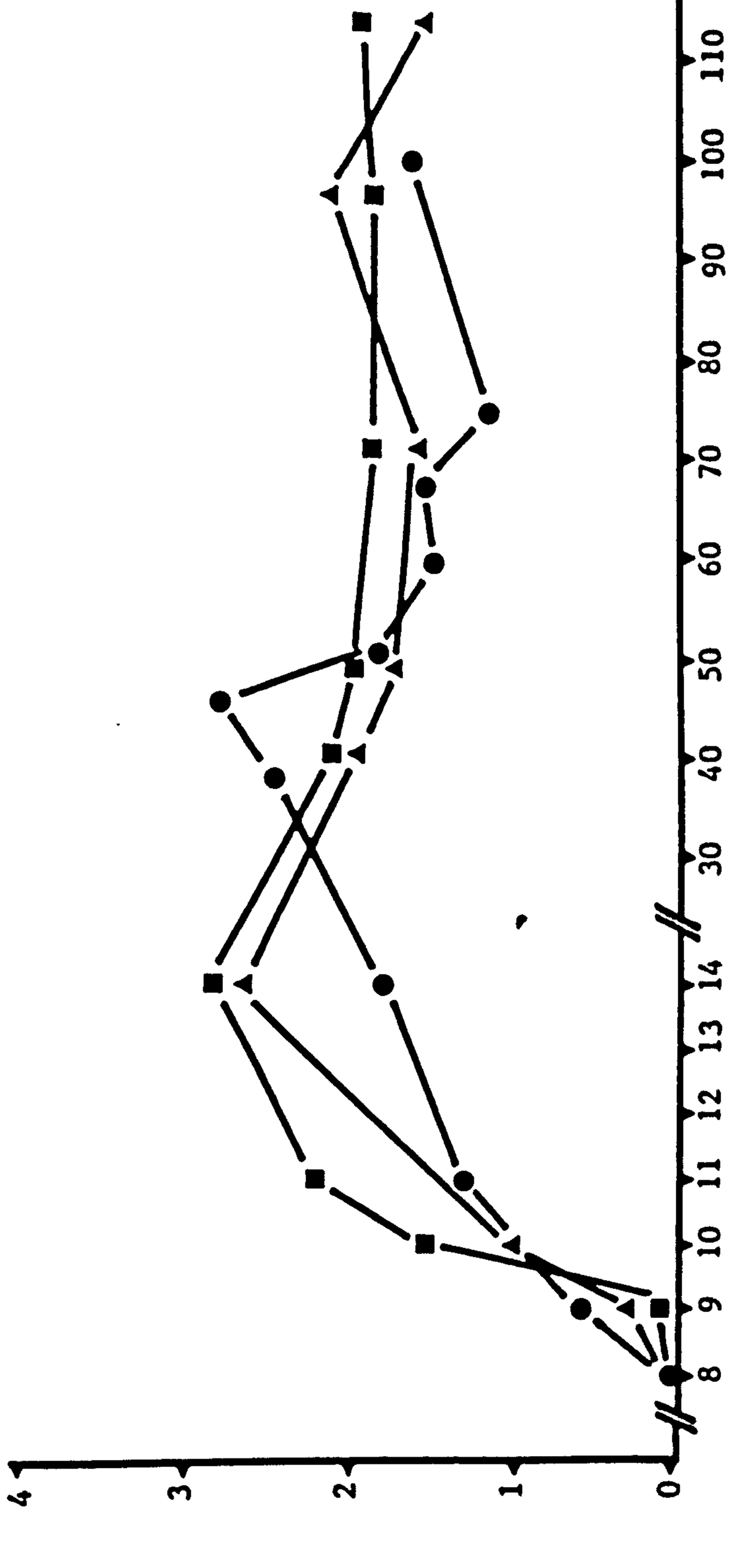


FIGURE 10. Onset and duration of conjunctival sensitivity to ovalbumin.

Three groups of guinea pigs were immunized with ovalbumin (500 µg) by intradermal (●-●: n = 11), intradermal (■-■: n = 10) and subcutaneous (▲-▲ : n = 10) injection. Conjunctival topical challenge with ovalbumin was determined by visual assessment in guinea pigs from each group between 8 and 14 days after immunization. All three groups were then subjected to further challenge at intervals during the subsequent 5-13 week period.

No significant decline in sensitivity was observed in any of the three groups as a result of regular topical challenge during the period 5-13 weeks after immunization. Furthermore, there was no significant difference in conjunctival sensitivity between the intradermal and subcutaneous injection groups.

1.9. Assessment of Conjunctival Reaction.

Four potential methods of conjunctival reaction assessment were compared; visual scoring, reflectometer readings, width of upper conjunctival swelling, and external conjunctival temperature. On each occasion, conjunctival reactions of increasing severity were elicited by intraconjunctival injection into the upper bulbar conjunctiva of a range of doses of histamine (1, 2, 5, and 10 µg). The reactions were routinely assessed 30 minutes after intraconjunctival challenge. The highly consistent nature of the histamine conjunctival response on different occasions allowed a meaningful comparison of data obtained over a series of experiments (Table 5).

Although appreciation and experience of the main symptoms of conjunctival reaction are required, visual appraisal of reaction severity within the 0 to 4+ system proved the quickest to perform, gave a clear increase of group mean score with dose, and resulted in small treatment standard errors. Both intraconjunctival challenge and reaction assessment were routinely performed 'blind' when using the visual scoring system, to eliminate any possibility of personal bias.

Measurement of conjunctival reaction intensity using reflectometer values was unsatisfactory. The guinea pigs required Evans Blue dye (20 mg/kg) by intravenous injection immediately prior to conjunctival

Table 5. METHODS OF ASSESSMENT OF THE CONJUNCTIVAL REACTION

INTRACONJUNCTIVAL Histamine Dose: µg	VISUAL ASSESSMENT 27.01.76	REFLECTOMETER DIFFERENCE mv. 9.10.75	CONJUNCTIVAL WIDTH mm. 9.10.75	REFLECTOMETER DIFFERENCE X WIDTH 9.10.75	TEMPERATURE PROBE °C 12.1.76
1	1.4 ± 0.1	2.5 ± 0.7	1.8 ± 0.2	4.6 ± 1.5	34.6 ± 0.2
2	1.8 ± 0.1	2.8 ± 0.5	2.4 ± 0.1	6.7 ± 1.2	34.0 ± 0.3
5	2.3 ± 0.2	2.9 ± 0.5	2.6 ± 0.2	7.4 ± 1.3	34.1 ± 0.3
10	2.8 ± 0.3	3.7 ± 0.4	2.2 ± 0.1	8.3 ± 1.2	n.d.

Guinea pigs were challenged on each occasion with increasing doses of histamine given by intraconjunctival injection. Reaction assessment was carried out at 30 minutes after challenge using the methods stated. Group sizes were ≥ 5 guinea pigs per treatment.

n.d. = Not done. mv. : millivolts. mm. : millimetres.

challenge. Readings took considerably longer to complete as both control and reacted eyes required testing to obtain a difference value for each experimental animal. The readings were also less consistent, giving larger group standard errors (Table 5) and a poor correlation with increased histamine dose (Table 6).

Measurement of the upper bulbar/palpebral conjunctival oedema using Vernier calipers resulted in consistent reaction scores except at the highest histamine dose level (10 µg). Oedema is sufficiently intense at this dose to begin to close the eye (Figure 3: 4+ reaction) making measurement difficult.

No improved correlation with histamine dose was obtained by calculating the value of the product of reflectometer difference and conjunctival oedema width for each guinea pig, when both methods were used in the same experiment.

In the experiment designed to test for variation in external conjunctival temperature using a thermistor temperature probe, no significant differences were observed between any treatment groups.

1.10 Repeated Conjunctival Challenge at Differing Time Intervals.

Three types of experiment were performed to investigate the effect of repetitive topical conjunctival challenge in guinea pigs with standard doses of ovalbumin (500 µg), histamine (250 µg), and compound 48/80 (7.5 mg). Groups of guinea pigs were challenged in the same eye at short, medium, and long time intervals of two, four, and twenty four hours respectively. Each eye was assessed immediately prior to challenge for residual symptoms, and at 30 minutes after the subsequent challenge.

Table 6: Correlation of reaction assessment with increasing intraconjunctival histamine dose.

Assessment Method	R	D.F.	Significance
Visual score	0.74	30	p<0.001
Reflectometer difference (R.D.)	0.38	18	0.1>p>0.05
Conjunctival width (C.W.)	0.17	18	n.s.
R.D. x C.W.	0.46	18	0.1>p>0.05
Conjunctival temperature	-0.24	22	n.s.

Correlation coefficient values (R) were derived from the data obtained for each conjunctival reaction assessment method presented in Table 5.

D.F.: Degrees of Freedom.

n.s.: Not significant.

In the first two experiments, guinea pigs were challenged four times between 10 a.m. and 4 p.m. at two hour intervals (Figure 11). A progressive increase in reaction severity with each successive challenge occurred in the ovalbumin challenged groups. Recovery was never complete between successive challenges which indicated that this might be an additive effect. There was therefore a highly significant difference between the first and fourth ovalbumin challenge responses ($0.005>p>0.001$). The histamine and compound 48/80 induced conjunctival reactions remained of similar strength with each challenge, and recovery from symptoms between challenges was almost complete.

Conjunctival challenge every four hours over a period of twenty hours produced a different picture (Figure 12). Compound 48/80 sensitivity was initially poor (1 + reactions), but remained constant with each successive challenge. Ovalbumin and histamine both elicited strong responses at the first challenge, but showed a gradual decline in reaction severity thereafter. Activity was therefore significantly reduced in both cases by the fifth and sixth challenges after 16 and 20 hours ($p<0.05$). Topical challenge with the same agents was performed in the contralateral eye of each animal four hours after the end of the experiment. The responses were slightly depressed, but not significantly different from those recorded in the first challenge.

Long term challenge was repeated every 24 hours for five days. A progressive decline in conjunctival response to all three types of challenge was observed (Figure 13). Ovalbumin and compound 48/80 reactions showed the sharper decline, being poor or absent as early as the second and third days. The histamine response decreased progressively to a 1 + response by the fifth day. Contralateral eye challenges

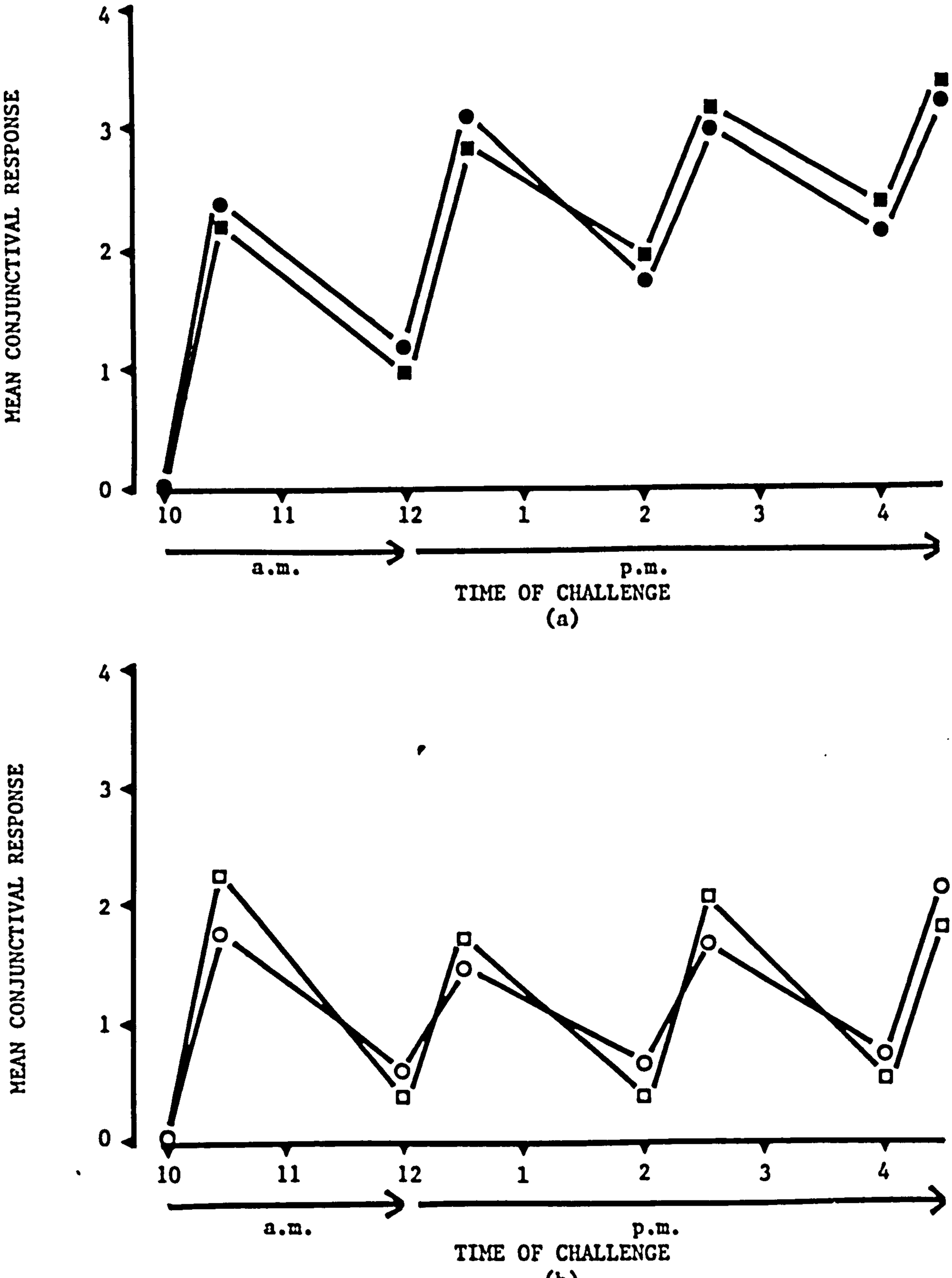


FIGURE 11. Repeated conjunctival challenge at two hour intervals.

In a series of experiments, four groups of guinea pigs were topically challenged with (a) ovalbumin (● - ● : n = 6; ■ - ■ : n = 8) in two cases, and (b) with either histamine (□ - □ : n = 8) or compound 48/80 (o - o : n = 8). Topical doses of ovalbumin, histamine and compound 48/80 were 500 µg, 250 µg and 7·5 mg respectively. In each experiment, the guinea pigs were topically challenged four times with one of these agents at intervals of 2 hours. Reactions were visually assessed, and residual symptoms from the preceding challenge were monitored in each case before the subsequent challenge.

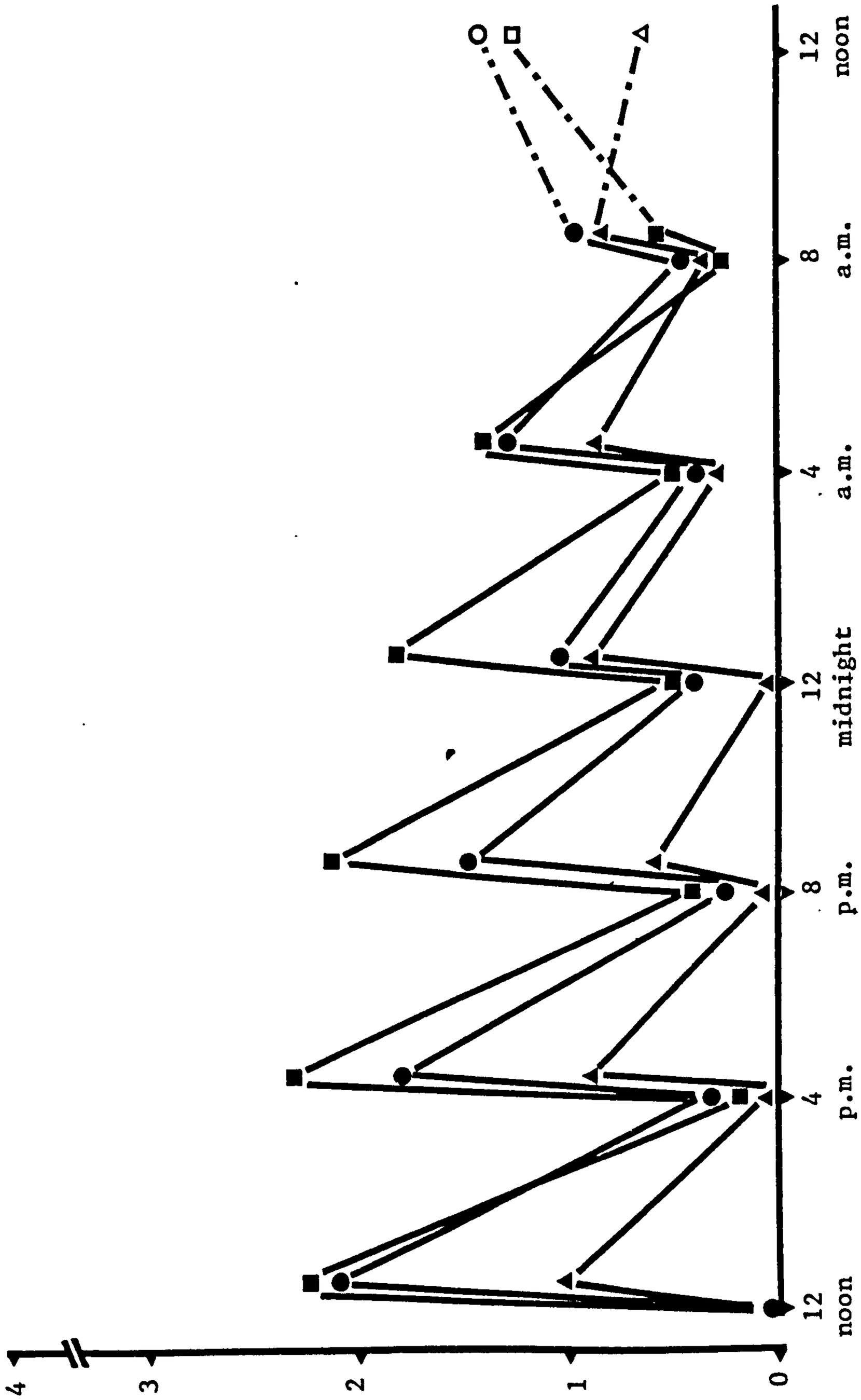


FIGURE 12. Repeated conjunctival challenge at four hour intervals.

Three groups of guinea pigs were topically challenged with ovalbumin (●-● : n = 5), histamine (■-■ : n = 8) six times at intervals of four hours. Topical doses of and compound 48/80 (▲-▲ : n = 8) were 500 µg, 250 µg and 7·5 mg respectively. Reactions were visually assessed, and residual symptoms from the preceding challenge monitored in each case before the subsequent challenge. Four hours after completion of the experiment, guinea pigs were also topically challenged in the contralateral eye with histamine (□-·-·-□) and compound 48/80 (Δ-·-·-Δ) as appropriate.

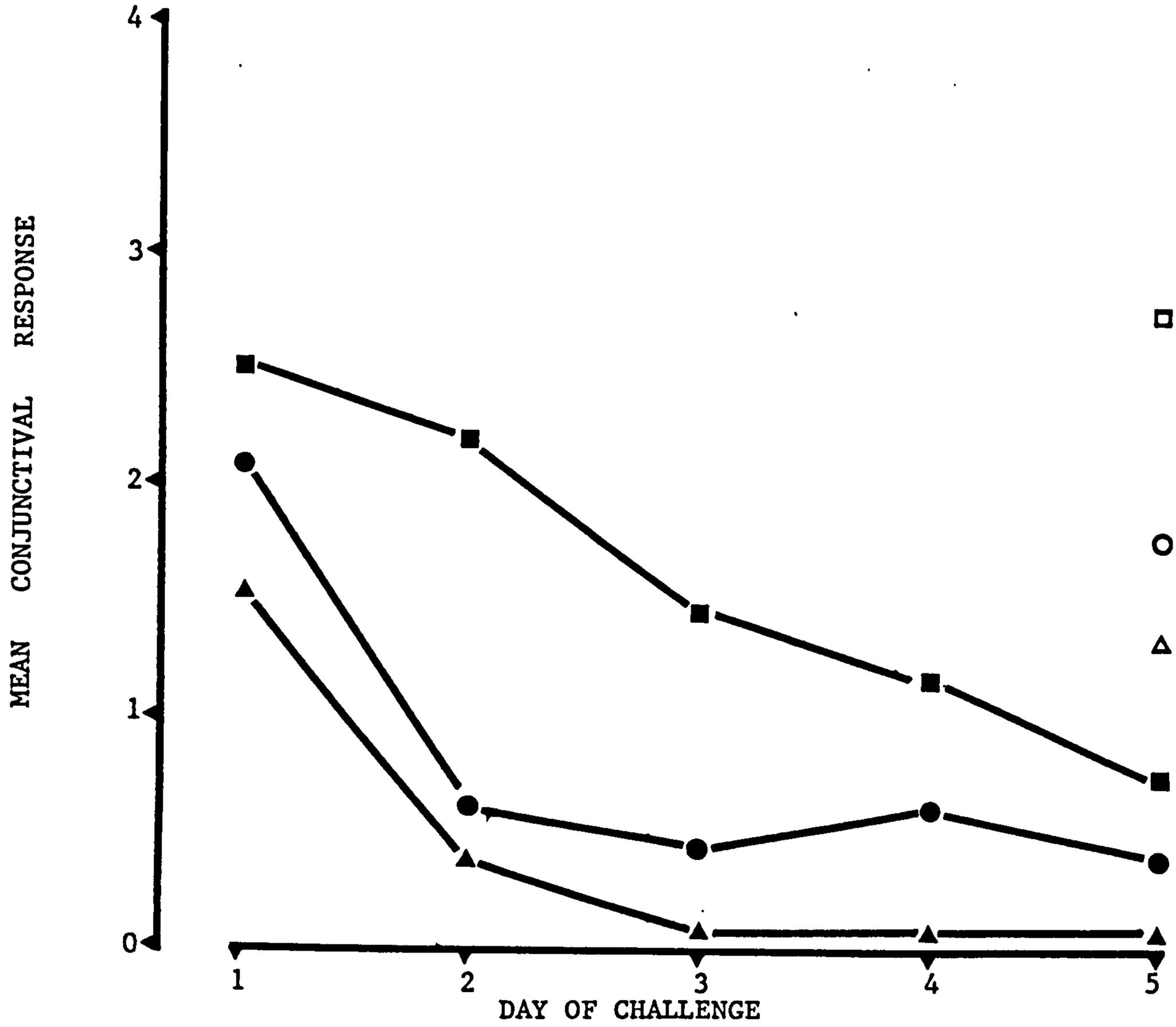


FIGURE 13. Repeated conjunctival challenge at twenty four hour intervals.

Three groups of guinea pigs were topically challenged with ovalbumin (● - ● : n = 8), histamine (■ - ■ : n = 9) and compound 48/80 (▲ - ▲ : n = 8) five times at twenty four hour intervals. Topical doses of ovalbumin, histamine and compound 48/80 were 500 µg, 250 µg and 7·5 mg respectively. Reactions were visually assessed. On the fifth day, guinea pigs in each group were also challenged in the contralateral eye with ovalbumin (○), histamine (□) and compound 48/80 (△) as appropriate.

on the fifth day produced conjunctival reactions of similar strength to those observed on the first day.

1.11. Variation in Antigen Sensitivity amongst different batches of guinea pigs.

Although all guinea pigs tested responded strongly to topical histamine challenge (250 µg), considerable variation was observed in the degree of antigen sensitivity shown by batches of animals obtained from different sources.

Only Charles River guinea pigs regularly showed strong primary (8th day) and secondary (accelerated) systemic reactions to serum antigens (see Table 1). Conjunctival sensitivity to topical rabbit serum challenge also proved strongest in guinea pigs obtained from Charles River (Table 7), and subsequent batches responded consistently to ovalbumin.

Redfern and Chelsea (Beecham) guinea pigs became strongly sensitive to ovalbumin following standard intradermal immunization, but responded poorly to serum antigens. Neither group showed systemic or topical reactions of any note. A single group of Tuck guinea pigs showed appreciable conjunctival sensitivity to rabbit serum in three of seven animals immunized, but only weak systemic reactions were observed.

Porcellus guinea pigs responded well to ovalbumin immunization, but only weakly to rabbit serum, while those obtained from Pirbright gave weak conjunctival reactions following ovalbumin immunization, but were not tested with serum antigens.

Table 7: Variation in antigen sensitivity amongst different batches of guinea pigs.

Challenge	Guinea Pigs	n	Conjunctival response % positive	Conjunctival response sensitivity (mean):
N.R.S.: (2 drops)	Charles River	15	40.6	2.0
	Charles River (boosted)	12	75.0	1.7
	Redferns	5	0	0
	Chelsea Stock	7	57.1	0.5
	Tuck	7	42.8	1.7
	Porcellus	5	60.0	1.0
OVA.: (500 µg)	Charles River	20	100.0	2.8
	Redferns	9	88.8	2.1
	Chelsea Stock	11	100.0	2.4
	Pirbright	40	87.5	1.3
	Porcellus	5	100.0	2.6
HIS.: (250 µg)	Charles River	9	100.0	2.1
	Redferns	9	100.0	2.2
	Chelsea Stock	15	100.0	2.6
	Pirbright	17	100.0	2.6

Male guinea pigs obtained from the above suppliers were immunized with rabbit serum (N.R.S.) or ovalbumin (OVA.) by intradermal injection, and challenged with these antigens or histamine (HIS.) as stated. Positive responders (%) and conjunctival sensitivity (mean) are shown for each batch of guinea pigs and type of challenge.

1.12. Species Variability of Conjunctival Reaction.

The effect of topical conjunctival challenge with antigen, histamine, 5-hydroxytryptamine, and compound 48/80 was investigated in six strains of mice, six strains of rats, four additional inbred strains of guinea pigs, and Chelsea stock rabbits. The results are summarised in Table 8.

Three strains of mice (LACA, CBA, and BALB/c) were immunized with rabbit serum (0.1 ml of a 5% solution) or ovalbumin (250 µg) by subcutaneous injection. No reaction was observed following topical conjunctival challenge with one drop of either rabbit serum or ovalbumin solution (10 mg/ml) in any of the mice tested, although the same mice proved strongly sensitive to pinnal antigen challenge performed as a positive control (Feinberg, 1961; Feinberg and Hill, 1968). The same mice, and also a further three strains (C57L, A2G, and NZB), failed to respond to topical challenge with histamine (100 µg) and 5-hydroxytryptamine (150 µg). Mice of the LACA, CBA, and BALB/c strains also showed no evidence of conjunctival oedema or erythema following instillation of compound 48/80 (2.0 mg).

Rats from only one strain (CFHB-Wistar) were immunized with antigen (0.02 ml rabbit serum or 250 µg ovalbumin by intradermal injection) and challenged after 2-3 weeks. No symptoms of conjunctival reaction resulted. Subsequent topical challenge of these same rats with a high dose of compound 48/80 (2.0 mg) produced some irritation, watering of the eye, and a little swelling of the eyelids. No intense erythema and oedema characteristic of the guinea pig conjunctival response was observed. These and five additional strains of rats (WA, AGUS, PVGC, WAG, and LH) were tested for conjunctival sensitivity to histamine

(150 µg) and 5-hydroxytryptamine (225 µg). No response was observed in any of the strains following histamine challenge. In two of the six strains (WAG and LH) distinct swelling of the eyelids and conjunctiva developed between 5 and 15 minutes after challenge with 5-hydroxytryptamine.

The variation in antigen sensitivity observed in outbred Hartley strain guinea pigs obtained from different outside suppliers has already been described. A limited amount of work with four additional strains of guinea pigs (R9, B, OM3, and complement C4 deficient) indicated that all were ovalbumin, histamine, and compound 48/80 sensitive where tested.

No conjunctival reactions were observed in New Zealand White rabbits following conjunctival challenge with antigen (ovalbumin), histamine, or 5-hydroxytryptamine. Sensitivity to compound 48/80 was not tested in the rabbit.

Table 8: Species Variability of the Conjunctival Response.

Species	Strain	Conjunctival challenge			
		Antigen	Histamine	5-HT	Comp. 48/80
Mouse:	LACA	-	-	-	-
	CBA	-	-	-	-
	BALB/c	-	-	-	-
	C57L	nd.	-	-	nd.
	A2G	nd.	-	-	nd.
	NZB	nd.	-	-	nd.
Rat:	CFHB	-	-	-	±
	WA	nd.	-	-	nd.
	AGUS	nd.	-	-	nd.
	PVGC	nd.	-	-	nd.
	WAG	nd.	-	+	nd.
	LH	nd.	-	+	nd.
Guinea pig:	Hartley	+	+	±	+
	R9	nd.	+	±	nd.
	OM3	nd.	+	±	nd.
	B	nd.	+	±	nd.
	C4 def.	+	+	nd.	+
Rabbit:	N.Z. White	-	-	-	nd.

Animals of the above species and strains were immunized and topically challenged with antigen (ovalbumin), histamine, 5-HT, or compound 48/80 as stated. Challenge volumes were: guinea pigs 25 µl; rats 15 µl; and mice 10 µl. Results were expressed as strong positive (+), weak positive (±) and nil (-) responders respectively.

nd. = not done

D I S C U S S I O N

The systemic erythematous and oedematous reactions which were observed following primary or secondary immunization with serum antigens closely resembled those previously described by Feinberg, Dewdney, and Temple (1965). Primary reactions were recorded in 47% and 13% of rabbit and bovine serum immunized guinea pigs respectively (Table 1), compared with a 40-70% incidence reported by the above authors. Secondary (accelerated) systemic reactions were observed in 100% and 67% of rabbit and bovine serum sensitive animals. No systemic reactions (primary or secondary) were seen at any time during experiments with sheep serum. Similar systemic reactions have also been reported in guinea pigs presensitized with aqueous extract and challenged with alum-precipitated pyridine-extracted preparations of timothy pollen (Chopra, Feinberg, and Chopra, 1968) and house dust mite (Chopra and Vincent, 1974).

Examination of blood smears taken prior to, during, and following systemic reactions to rabbit serum confirmed the presence of sequential rises in eosinophil (days: S, S+I) and basophil (days: S+II, S+III) counts (Table 2), although these increases were less extreme than those previously described (Feinberg, Dewdney, and Temple, 1965).

The severity of the conjunctival response to topical serum challenge appeared to be closely linked with the incidence of primary and secondary systemic reactions (Table 1). Absence of a systemic sensitization reaction did not preclude conjunctival reaction, but in such cases the conjunctival response was invariably weak, particularly in the sheep serum immunized group. The symptoms of immediate conjunctival hypersensitivity in guinea pigs have been previously described

(Feinberg and Chopra, 1966; Dwyer and Darougar, 1971; Dwyer, Turk, and Darougar, 1974).

Sensitization of guinea pigs to protein antigens proved straightforward and successful. Guinea pigs immunized with ovalbumin by intradermal injection generally responded with 2-3+ conjunctival reactions at first challenge, and there were few poor or nil responding animals in each batch. The route of injection used for protein antigen immunization may also not be important. For example, immunization by either intradermal or subcutaneous injection resulted in strong conjunctival sensitivity in the present study. Larger ovalbumin doses (60 mg) given intraperitoneally and subcutaneously have previously proved equally successful (Taylor and Roitt, 1973). The lyophilized bacterial amylase protein (purified from B. subtilis) had been originally employed for aerosol sensitization and inhalation challenge in guinea pigs by Yamamura, Yagura, and Miyuke (1973). In the present investigation, notably strong conjunctival responses were obtained following immunization with this protein.

Comparison of the conjunctival sensitivity which resulted from immunization with either serum or protein antigens suggested several important advantages in the use of the latter. Conjunctival sensitivity to ovalbumin was initially strong, long lasting, and required no booster injections (Figure 10). In contrast, the response following immunization with serum antigens was less consistent, required routine secondary injections, and appeared to be guinea pig batch dependent (Tables 1 and 7).

The incidence and severity of an allergic response in man is now known to be predominantly governed by genetic considerations (see

General Introduction). The possibility that genetic variation between strains of guinea pigs might affect the strength of the conjunctival response, (as implied by the data in Table 8), is further supported by the observations of Ovary, Kaplan, and Kojima (1976). These authors studied the anti-dinitrophenyl (DNP) IgE antibody response to immunization with DNP-Ascaris extract in guinea pigs. Of those strains tested, Hartley strain gave the lowest titres, while substantially higher serum IgE levels were recorded in English Short Hair strain guinea pigs. Unfortunately, the latter strain was not tested during the present investigation.

The importance of ensuring a consistent and accurate topical antigen dose was strongly emphasised by the dose-response relationship observed with ovalbumin challenge (Figure 5); an effect more difficult to control but presumably equally important for serum antigens. The identification of the specific serum components antigenically active in the conjunctival response was therefore felt to be a first step towards purification of antigenic material. However, little evidence was obtained to indicate that the major serum proteins are active antigenic constituents of serum antigens with regard to immediate conjunctival hypersensitivity. None of the bovine serum sensitive guinea pigs showed a positive conjunctival response when tested with bovine serum albumin or gamma globulin. Furthermore, only 2 of 5 rabbit serum sensitive animals reacted when challenged with pooled fractions of rabbit serum separated by gel diffusion chromatography which corresponded to the main protein elution peaks, although additional antigenic material may have been present in the untested fractions eluted between these peaks.

Histamine is an established anaphylactic mediator in the guinea pig.

It's ability to reproduce anaphylactic type symptoms, and the effectiveness of specific histamine antagonists in preventing them, has been demonstrated in guinea pig lung (Auer and Lewis, 1910; Armitage, Herxheimer, and Rosa, 1952; Brocklehurst, 1960; Popa, Douglas, and Bouhuys, 1973; Drazen and Austen, 1974), trachea (Constantine, 1965; Coleman and Farmer, 1971; Weiss, Anderson and O'Brien, 1975), skin (Miles and Miles, 1952; Yeoh, Tay, and Greaves, 1972), and ileum (Joiner, Wall, Davis, and Hahn, 1974).

The effect of topical histamine instillation onto the guinea pig conjunctiva, previously unreported, was both dramatic and pronounced. The conjunctival reactions became evident as early as 2-3 minutes after challenge, and were frequently of equal or greater severity than those resulting from antigen challenge.

In contrast, the response to topical or intraconjunctival challenge with 5-hydroxytryptamine was poor, even at dose levels up to 300 times greater than those at which histamine was active. This result is consistent with previous reports that guinea pig tissues including skin and ileum, although not lung, are substantially less sensitive to 5-hydroxytryptamine than those of the rat (Herxheimer, 1955; West, 1957; Sanyal and West, 1958; Mongar and Schild, 1962; Collier and James, 1967; Maling et al., 1974).

The ability of compound 48/80 to release histamine from tissue mast cells has been extensively investigated in dogs (Feldberg and Talesnik, 1953), cats (Paton, 1951; Westerholm, 1960), guinea pigs (Mongar and Schild, 1952; Mota and Vugman, 1956a, 1956b; Feinberg and Sternberger, 1955), and rats (Moran et al., 1962; Horsefield, 1965; Ranadive and Ruben, 1973).

Many workers have compared the mechanisms by which antigen and compound 48/80 induce release of histamine from isolated rat peritoneal mast cells. In common with antigen challenge, compound 48/80 release requires energy supplied by glycolysis and oxidative phosphorylation (Diamant and Uvnas, 1961; Thon and Uvnas, 1967; Peterson, 1974b), and can be inhibited by agents such as isoprenaline which are known to increase intracellular levels of cAMP (Hogberg and Uvnas, 1960; Loeffler, Lovenberg, and Sjoerdsma, 1971; Johnson, Moran, and Mayer, 1974; Fredholm et al., 1976). Unlike antigen release however, compound 48/80-induced secretion of histamine from rat mast cells is still able to proceed in a calcium free medium (Uvnas, 1968). This latter release is inhibited by preincubation with EDTA, and is thought to indicate the use of tightly bound intracellular calcium (Dahlqvist, Diamant, and Elwin, 1973; Kagayama and Douglas, 1974; Cochrane and Douglas, 1974; Kruger, 1976).

There is some evidence available to suggest that the *in vivo* activity of compound 48/80 in the guinea pig may not be entirely due to release of histamine from mast cells, as is thought to be the case in the rat or dog. Feinberg and Sternberger (1955), despite finding variation in potency between batches, reported that all samples of compound 48/80 tested produced severe symptoms of itching, sneezing, retching, and prostration in guinea pigs given intraperitoneal injections of 3 mg/kg. However, both injection and inhalation of compound 48/80 failed to produce the severe broncho-constriction characteristic of systemic guinea pig anaphylaxis. The view of the above authors that compound 48/80 may have additional toxic side effects in the guinea pig supported the earlier conclusions of Miles and Miles (1952). These authors showed that although 2-4 µg intradermal injections of compound

48/80 cause visible cutaneous permeability reactions in pontamine blued guinea pigs, extensive thrombotic damage to blood vessels is also apparent. Furthermore, there is no change in the microscopic appearance of guinea pig lung, skin, or mesenteric mast cells following intra-peritoneal injection of compound 48/80 (Mota and Vugman, 1956a, 1956b).

Clearly therefore, the conjunctival reactions observed in response to high topical and intraconjunctival doses of compound 48/80 during the present investigation may have resulted from other local non-specific toxic or inflammatory effects, in addition to mast cell amine release.

The divalent cation ionophore A23187 (Chaney, Demarco, Jones and Occolowitz, 1974) has been shown to induce granule exocytosis and histamine release from rat mast cells (Foreman, Mongar, and Gomperts, 1973) and human basophil leucocytes (Lichtenstein, 1975) *in vitro*. These and other studies have demonstrated that this release is dependent on an ionophore facilitated transmembrane flux of calcium ions into the mast cell or basophil (Foreman and Mongar, 1972; Foreman, Gomperts, and Mongar, 1973).

There have been few reports of experiments designed to test the activity of A23187 *in vivo*, although its ability to release both histamine and SRS-A from rat peritoneal mast cells following intra-peritoneal injection has been described (Bach and Brashler, 1974). Intraconjunctival doses of A23187 (30-100 µg) elicited typical oedamatous and erythematous conjunctival reactions in the present study. However, the precise *in vivo* effects of an agent which is unable to descriminate between different divalent cations or cell types must be

subject to further investigation.

No response was obtained in guinea pigs to intraconjunctival doses of prostaglandins E_1 and E_2 (100 ng - 10 µg) during the present investigation, although some evidence of minor irritation was apparent following injection of the higher PGE_1 doses. While it has been shown that PG's are released from antigen challenged guinea pig lung (Bakhle and Smith, 1972; Piper and Vane, 1969, 1971; Yen, Mathé, and Dugan, 1976) and trachea superfused with histamine (Orehek et al., 1973; Grodzinska et al., 1975), this species appears less sensitive in some respects to the effects of E series PG's than the rat. For example, PGE_1 and PGE_2 both cause increased vascular permeability when injected intradermally into rats (Crunkhorn and Willis, 1969; Freeman and West, 1972; Thomas and West, 1974). This effect is probably mediated via mast cells, in view of the inhibitory activity of mepyramine, methysergide, and $PGF_{2\alpha}$ against the response (Crunkhorn and Willis, 1971a, 1971b). In contrast, only weak cutaneous responses are observed following PGE_1 injection in the guinea pig (Horton, 1963; Williams and Morley, 1973).

The results obtained during study of the effect of repetitive topical conjunctival challenge in guinea pigs raised several quite interesting questions, while at the same time offering few solutions. The short term (two hour interval) challenge responses were found to be of either constant (histamine and compound 48/80) or increased (ovalbumin) strength. In contrast, when challenge was performed at four or twenty four hour intervals, conjunctival responses to both ovalbumin and histamine exhibited a sequential decline. In the case of repeated challenge at the two longer time intervals, loss of sensitivity occurred

only in the conjunctiva subjected to repeated challenge. Testing of the contralateral eye at the conclusion of each experiment invariably resulted in normal strength conjunctival responses.

The short term interval challenge data shown in Figure 11 demonstrated the useful and important possibility of sustaining a conjunctival response to antigen or histamine over a period of several hours. This type of response may be of more direct relevance to the clinical situation in man than a single dose acute reaction. The gradual decline in antigen sensitivity observed with longer interval antigen challenge might be due to depleted mast cell amine stores, although a reduction in histamine sensitivity also occurred. Agonist receptor saturation may also contribute to the loss of sensitivity to both agents. Most important of all might be the infiltration into the conjunctival tissue of substantial numbers of neutrophils and eosinophils (as described in Chapter Four of this thesis) if these cells are involved in the inactivation of anaphylactic mediators and phagocytosis of immune complexes. The fact that these considerations do not appear to apply to the short term interval challenge experiment could perhaps be due to partially excited tissues remaining sensitive to additional challenge, whereas recovered tissues may go through a refractory phase. Further work is clearly required to elucidate these points more fully.

The conjunctival sensitivity demonstrated in two strains of rats (Wistar albino and Liverpool hooded) to 5-hydroxytryptamine but not histamine is in agreement with previous work emphasising that the former agent is an important anaphylactic mediator in this species (Sanyal and West, 1958; Maling et al., 1974). The greater sensitivity of rats to intradermal PGE_1 reported by Crunkhorn and Willis (1969, 1971a,

1971b) also suggests that the conjunctiva of the rat might be more responsive to PGE_1 than that of the guinea pig.

The only previous report to the knowledge of the author of an immediate hypersensitivity reaction in the conjunctiva of a species other than the guinea pig or man, is that in the rabbit described by Abdel-Maguid, Nofal, and Hennawi (1973). In contrast to the present study, in which no reactions were seen following topical challenge with antigen, histamine, or 5-hydroxytryptamine in the rabbit, the above authors obtained positive responses to intraconjunctival injection of ovalbumin (immunized animals) and histamine. The discrepancy between the two studies remains to be resolved, but might be explained by the difference in route of challenge employed.

CHAPTER TWO

SERUM ANTIBODY STUDIES

IN SENSITIZED GUINEA PIGS

I N T R O D U C T I O N

The observations of Yagi, Maier, and Pressman (1962), White, Jenkins, and Wilkinson (1963), and Benacerraf, Ovary, Bloch, and Franklin (1963), led to the recognition of two distinct 7S IgG antibody populations in sensitized guinea pigs. These antibody populations were shown to possess different electrophoretic mobilities in agar gel and starch block systems, could be cleanly separated by ion exchange chromatography on DEAE cellulose, and were appropriately designated as electrophoretically fast IgG_1 (gamma-1) and slow IgG_2 (gamma-2).

While IgG_1 antibodies appeared capable of mediating passive local or systemic anaphylactic reactions, IgG_2 antibodies did not (Ovary, Benacerraf, and Bloch, 1963; Baker, Bloch, and Austen, 1964; Stechschulte, Austen, and Bloch, 1967). In contrast, IgG_2 but not IgG_1 antibodies fixed complement by the classical nine component pathway, demonstrated by lysis of tanned antigen-coated sheep red blood cells (Bloch, Kourilsky, Ovary, and Benacerraf, 1963). Although a later report has shown that pre-prepared, washed, specific IgG_1 antibody-antigen precipitates are able to fix complement and generate anaphylatoxin by a third component (C'3) bypass mechanism (Osler, Oliveira, Shin, and Sandberg, 1969), the involvement of complement in guinea pig anaphylactic reactions is doubtful. For example, IgG_1 mediated passive cutaneous anaphylaxis (PCA) in this species is not inhibited by an anti-complementary factor isolated from cobra venom (Cochrane, Muller-Eberhard, and Aikin, 1970).

Homocytropic anaphylactic antibody activity in the guinea pig was therefore primarily associated in the early 1960's with the 7S IgG_1

(gamma-1) class of antibody. The characterization of reaginic antibody activity in man as belonging to a new homocytotropic immunoglobulin class termed IgE (Ishizaka, Ishizaka, and Hornbrook. 1966; Ishizaka and Ishizaka, 1967) coincided with preliminary evidence for the existance of IgE-like antibodies in rats and mice subjected to helminth infection (Mota 1964; Ogilvie 1967; Revoltella and Ovary, 1969). The presence of homocytotropic reaginic antibodies was subsequently demonstrated in the mouse (Levine and Vaz, 1970), rat (Stechschulte, Orange, and Austen, 1970), guinea pig (Catty, 1969), and rabbit (Zvaifler and Robinson, 1970).

In the guinea pig, as in these other experimental animals, reaginic (IgE) antibody was principally identified by its ability to passively sensitize skin sites following local serum transfer for long periods of time (> 8 days). This activity could be destroyed by heat (56-60°C) and 2-mercaptoethanol treatment as in man, and was thus easily distinguished from the short term sensitizing, heat and 2-mercaptoethanol stable 7S IgG_1 antibodies previously described (Catty, 1969; Mota and Perini, 1970; Levine, Chang, and Vaz, 1971). Despite these early observations, the chromatographic isolation, purification, and subsequent description of the physicochemical and biological properties of guinea pig IgE has only recently been achieved (Margni and Hajos, 1973a, 1973b).

In addition, other reports have proposed the existence of a third homocytotropic antibody in the guinea pig. This is thought to be a second type, or sub-population, of IgG_1 type antibody, and is heat stable, but sensitive to 2-mercaptoethanol treatment. It is reported to be partially separable from the 2-mercaptoethanol stable IgG_1 using DEAE cellulose ion exchange chromatography, and shows maximal mast cell sensitizing activity in PCA tests at 2-4 day latent periods (Parish,

1970c; Ovary and Warner, 1972; Perini and Mota, 1972). Active or passive anaphylactic reactions in the guinea pig may therefore be attributable to one or more of three types of homocytotropic antibody, two IgG_1 sub-populations and IgE, possessing short, medium, and long term mast cell sensitizing activity respectively.

The nature of the anaphylactic antibodies active in animal models of allergic conditions is especially important in view of the finding that the inhibitory activity of known anti-allergic agents such as disodium cromoglycate may depend on the class of homocytotropic antibody (IgG_1 or IgE) actively or passively sensitizing mast cells for mediator release (Goose and Blair, 1969; Orr, Gwilliam, and Cox, 1970, 1971; Taylor and Roitt, 1973).

The object of the present work was therefore firstly, to characterize the serum antibodies produced in response to antigen immunization for conjunctival anaphylaxis, and secondly, to monitor the effect of regular conjunctival challenge on those antibody activities detected. Antibody activity was investigated in four types of test comprising (i) PCA at increasing latent periods of sensitization with selective heat and 2-mercaptoethanol serum treatment, (ii) haemagglutination of antigen coated sheep red blood cells, (iii) haemolysis of antigen coated sheep red blood cells, and (iv) precipitin formation in agar gel plates.

MATERIALS AND METHODS

Animals:

Outbred albino Dunkin-Hartley strain guinea pigs obtained from Charles River U.K. Ltd., or Redfern Animal Breeders Ltd., were used for both immunization and PCA studies.

Antigens:

Ovalbumin, 5x recrystallized (Koch-Light Laboratories Ltd).

Rabbit serum (Wellcome Research Laboratories).

Immunization/Topical Challenge/Reaction Assessment:

All methods as described in Chapter One of this thesis.

Sera:

Blood was collected from guinea pigs either by cardiac puncture or at sacrifice, at various time intervals after immunization. After being allowed to clot (6-18 hours at 4°C), serum was taken off by centrifugation (200 g for 15 minutes) and stored frozen at -15°C until use. The serum samples used in the haemagglutination and haemolytic antibody tests were complement inactivated by heating at 56°C for 30 minutes.

Preparation of normal and ovalbumin-coated sheep erythrocytes:

Normal sheep red blood cells were obtained preserved in Alsever's solution (Wellcome Reagents or Oxoid) and washed three times with Dulbecco phosphate buffered saline (PBS) pH 7.4, before being packed by centrifugation (250 g for 15 minutes at 4°C). 0.3 ml aliquots of packed red cells were resuspended in 5 ml PBS, to which 5 ml of freshly made up tannic acid solution (0.1 mg/ml) was added. The cells were then incubated at 37°C for 15 minutes, centrifuged (200 g for 5 minutes at

4°C), and the supernatant taken off. A proportion of the cells were then put aside as tannic acid treated uncoated controls, while ovalbumin (5 ml of a 2 mg/ml solution) was added to the remainder, which were gently resuspended and incubated for a further 30 minutes at 37°C. All the cells (coated and uncoated) were then separately washed three times and resuspended in PBS containing 1% inactivated and absorbed rabbit serum.

Haemagglutinating antibody tests:

Control (tanned uncoated) and antigen (ovalbumin) coated sheep red cells (1% suspensions) were added to serial doubling dilutions of each serum prepared in MRC pattern agglutination plates (0.1 ml volumes of each). The contents of the wells were mixed by gently agitating the plates, and the agglutination reactions were scored on a visual 0 to 4+ basis after incubation for 18 hours at 4°C. The titre of each serum was taken as the highest dilution showing a 1+ result (i.e. the dilution at which first detectable positive agglutination of red cells occurred). Positive, negative, nil-serum, and uncoated tanned cell controls were included in all experiments.

Haemolytic antibody tests:

Tanned uncoated and ovalbumin coated sheep red cells were taken and serum dilutions prepared in MRC plates as in the haemagglutination test, using haemolytic diluent (Dulbecco A+B solutions). Freshly reconstituted preserved guinea pig serum diluted 1:30 was added to each well as a source of complement (0.1 ml per well). The plates were then incubated for one hour at 37°C, and visually assessed for lysis on a 0 to 4+ scale. Titres were quoted as the reciprocal of the serum dilution giving 50% (2+) lysis. Positive, negative, nil serum, and nil complement controls were included in each test.

Titres for both haemagglutinating and haemolytic antibody tests were expressed in $\log_2$ units.

Immunodiffusion:

Sera were tested for the presence of precipitating antibody activity by diffusion against antigen in New Zealand agar gel plates (Feinberg, 1957). The agar was prepared at 1% dilution, and poured in petri dishes to a depth of 3mm. Well patterns were cut using Shandon-Feinberg gel cutters. The sera were diffused against a range of ovalbumin solutions (10, 1, 0.1, and 0.01 mg/ml) and observed for the presence of precipitin lines after incubation for 48 hours at 4°C.

Passive Cutaneous Anaphylaxis (PCA):

PCA tests were performed essentially following the method of Ovary (1952, 1964). Sensitization of shaved guinea pigs was accomplished by 0.1 ml intradermal injections of serial dilutions of serum from ovalbumin sensitive guinea pigs spaced equally on either flank. No guinea pig received more than twelve injections in all, or more than six on either flank.

Various sensitization time intervals were used where stated to distinguish between different types of homocytotropic antibody. For the time course experiment, in which six latent periods of sensitization were used (4, 8, and 24 hours, 2, 4, and 8 days), the complete time course for any one serum sample was performed on the same guinea pig by sequential injections on one or other flank at the specified times. Control injections of normal diluted guinea pig serum were incorporated in all experiments.

Challenge was effected by a 1 ml/kg intravenous injection (ear

vein) of a solution 20 mg/ml in Evan's Blue, and 1.0 mg/ml in ovalbumin. The guinea pigs were sacrificed, skinned, and the reactions assessed at 30 minutes after challenge. The score for each reaction consisted of the arithmetic mean of two diameters measured at right angles. Although assessment of the intensity of blueing was also routinely recorded (visually 0 to 4+ scale, or with the reflectometer (see Chapter One)), statistical analysis of the results indicated that no additional information was provided by including a measure of blueing intensity.

Separation of 7S IgG_1 and IgG_2 guinea pig antibodies:

A sample of pooled guinea pig serum (see text) was chromatographed on Whatman DE-52 pre-swollen ion exchange cellulose following the method outlined by Fahey, McCoy, and Goulian (1958). A 2.0 ml sample was equilibrated with the starting buffer by overnight dialysis at 4°C, and the resultant faint precipitate was removed by centrifugation (2500 rpm for 15 minutes at 4°C). The sample was then loaded onto a 1.5 x 15 cm DE-52 column, and a potassium phosphate buffer molarity gradient of 0.01M (0.009M K_2HPO_4, 0.01M KH_2PO_4 pH 7.9) to 0.30M (0.282M K_2HPO_4, 0.018M KH_2PO_4 pH 7.9) passed across the column. A constant flow pump maintained a flow rate through the column of 0.5 ml per minute. 2.0 ml fractions were collected on an LKB 7000A Ultrorac fraction collector, and monitored for protein content by ultraviolet absorption on a Perkin-Elmer spectrophotometer at 280 nm.

Phosphate determination:

Phosphate ion concentration and thus buffer molarity was determined in selected fractions from the DE-52 separation using the following micro-determination method.

20 µl samples from every tenth fraction were made up to 1.0 ml with

distilled water in test tubes. 1.0 ml of 2% L-ascorbic acid solution in 10% trichloroacetic acid (w/v) was added to each tube, followed by 0.5 ml of a 1% ammonium molybdate solution. After two minutes, 1.0 ml of citrate-arsenite solution (2.0g sodium citrate.$2H_2O$ + 2.0g anhydrous sodium arsenite + 2.0 ml glacial acetic acid made up to 100 ml with distilled water) was added, and the tubes were allowed to stand at room temperature for 15 minutes while the colour reaction developed.

A standard curve for phosphate ion concentration was determined using known standards (0, 1, 2, 5, and 10 µg phosphate per ml). From their absorbance at 780 nm, the phosphate ion concentrations in each of the ten fractions were read off on the standard curve. The phosphate ion gradient across the DE-52 column was then calculated in terms of molarity.

Heat treatment of sera:

The pooled serum samples were placed in sealed tubes and heated in a water bath at 56°C for four hours (Perini and Mota, 1972).

2-mercaptoethanol treatment of sera:

Either pooled or DE-52 fractionated serum samples were dialysed against the same two litre volumes of 0.1M 2-mercaptoethanol (3 hours), and then 0.02M iodoacetamide (4 hours), at room temperature with stirring (Mota and Perini, 1970). The samples were then dialysed against four changes of PBS pH 7.4 over 24 hours at 4°C. Control samples were dialysed against PBS in place of 2-mercaptoethanol, and treated in the same way as test samples with iodoacetamide and four further PBS changes.

Statistics:

Conjunctival response scores were analysed using the Wilcoxon Rank Sum Test. All other results (PCA scores, antibody titres etc.) were analysed using Student's t-test. $p<0.05$ was taken as indicating a significant result in both cases.

R E S U L T S

2.1. Preliminary Studies on the Nature of Serum Antibodies in Conjunctivally Sensitive Guinea Pigs.

2.1.1. Initial PCA experiments:

A series of experiments were performed to investigate the homologous PCA activities of sera from guinea pigs actively sensitive in conjunctival challenge to rabbit serum or ovalbumin. Sera from five rabbit serum sensitive guinea pigs, and three groups of ovalbumin sensitive guinea pigs (6, 8, and 19 animals respectively) were tested over a range of fourfold serum dilutions (1:4, 1:16, 1:64 and 1:256), at two passive sensitization latent periods of 4 hours (short term) and 7 days (long term). All five of the rabbit serum sensitive guinea pigs had shown strong (3+) conjunctival reactions, while responses in the ovalbumin group had varied between 1+ and 4+.

The sera from all the guinea pigs showed strong short term (4 hour) PCA reactions at 1:4 and 1:16 dilutions, and weaker activity at 1:64 and 1:256 (Table 9). Although 7 day PCA activity was again strong in the sera from rabbit serum sensitive guinea pigs, it was weak or absent in the sera from ovalbumin immunized groups.

2.1.2. Correlation between serum PCA activity and conjunctival response:

To test for correlation between PCA reaction scores and conjunctival reaction severity, ten guinea pigs were immunized with ovalbumin in the normal way. Blood samples (approximately 2 ml) were taken from each animal by cardiac puncture at 14 and 28 days after immunization and sera prepared. PCA activity was determined for each serum sample at 4 hour and 7 day latent periods. These PCA scores were then compared

TABLE 9. SERUM PCA ACTIVITIES IN GUINEA PIGS IMMUNIZED WITH OVALBUMIN OR RABBIT SERUM.

IMMUNIZATION			SERUM P C A ACTIVITY mean reaction diameters ± s.e.m. (mm)							
			4 HOURS				7 DAYS			
ANTIGEN	DOSE	n	1:4	1:16	1:64	1:256	1:4	1:16	1:64	1:256
OVA	1·0 mg	6	12·9 ± 1·3	9·5 ± 1·1	5·5 ± 1·1	n.d.	0	0	0	n.d.
OVA	1·0 mg	8	11·2 ± 0·5	7·4 ± 0·8	0·9 ± 0·4	n.d.	0	0	0	n.d.
OVA	500 µg	19	17·8 ± 0·7	14·1 ± 0·6	9·8 ± 0·7	n.d	2·6 ± 1·1	0	0	n.d.
N.R.S	0·02 ml	3	18·0 ± 0·9	14·2 ± 1·0	8·9 ± 1·4	n.d	15·3 ± 2·1	10·6 ± 1·4	1·2 ± 1·1	n.d.
N.R.S	0·04 ml	2	n.d.	16·9 ± 0·1	13·8 ± 1·0	11·1 ± 0·9	n.d.	10·8 ± 1·0	5·6 ± 1·6	0

Guinea pigs were immunized with ovalbumin (OVA) or rabbit serum (N.R.S.) by intradermal injection. Serum was obtained at sacrifice between 28 and 56 days after immunization, following investigation of conjunctival sensitivity. PCA activities were determined in duplicate, for each serum sample, at each dilution and sensitization latent period (4 hours or 7 days). The results were expressed as group mean PCA reaction diameters (mm) ± standard error of the mean (s.e.m.).

n.d.: not done.

with conjunctival reaction severity following topical antigen challenge at 18, 25, and 34 days after immunization. The results are shown in Table 10.

Short term PCA activity proved poor in the sera obtained at 14 days, but significantly stronger at 28 days ($0.01>p>0.005$). Long term (7 day) PCA activity was nil in all of the samples. There was no significant difference between the conjunctival responses recorded on days 18, 25, or 34 (group mean scores ± s.e.m.: 2.3 ± 0.3, 1.7 ± 0.2, and 2.0 ± 0.3 respectively). There was no significant positive correlation between 4 hour PCA scores and conjunctival reaction severity, although a significant inverse (negative) correlation resulted between 25 day conjunctival challenge and 28 day PCA activity (Table 11).

2.1.3. Placental transfer of conjunctival sensitivity:

An experiment was designed to study the placental transfer (if any) of conjunctival sensitivity from parent guinea pigs to offspring. Eight pregnant guinea pigs were immunized approximately six weeks before term with either ovalbumin (5 animals) or rabbit serum (3 animals). After a further 14 days all eight showed positive conjunctival responses to their respective antigens, although none greater than 2+. Within 24 hours of the birth of each litter, both mother and young were topically challenged with either ovalbumin or rabbit serum as appropriate. In two of the five litters from ovalbumin immunized mother guinea pigs (7 young in all), weak but unmistakeable conjunctival responses were observed. The transfer of ovalbumin sensitivity to offspring appeared to follow an inverse relationship, with the least sensitive guinea pigs giving birth to responding litters (Table 12). No reactions were recorded in any of the litters born to rabbit serum immunized guinea pigs, although the mothers responded normally to challenge.

Table 10. Comparison of conjunctival response severity and serum PCA activity (fourfold dilution at four hours) in ovalbumin sensitive guinea pigs.

Guinea pig.	14 day PCA.	18 day C.R.	25 day C.R.	28 day PCA.	34 day C.R.
1.	3.5	2.0	1.5	17.5	2.0
2.	n.d.	2.5	2.0	15.3	2.0
3.	0	2.5	1.5	n.d.	2.0
4.	11.0	3.0	0.5	18.0	n.d.
5.	2.3	0	2.5	14.3	2.0
6.	5.8	2.0	1.0	18.8	1.0
7.	14.3	2.5	1.0	16.5	0.5
8.	17.5	2.0	1.5	17.5	2.5
9.	11.8	3.5	3.0	14.3	3.0
10.	0	3.0	2.0	17.0	3.0

Passive cutaneous anaphylaxis (PCA) scores for sera obtained by cardiac puncture at 14 and 28 days after intradermal immunization with ovalbumin are expressed as the mean reaction diameter in mm. for each serum sample determined in duplicate. The conjunctival response (C.R.) was scored visually (0 to 4+ scale). Correlation coefficients between variables are shown in Table 11.

n.d.: not done

Table 11. Correlation coefficient analysis between the severity of conjunctival response and short term (4 hour) serum PCA activity.

Correlation test				
Variable 1	Variable 2	R	D.F.	P
14 day PCA	18 day C.R.	0.25	7	n.s.
14 day PCA	25 day C.R.	0.21	7	n.s.
28 day PCA	25 day C.R.	-0.84	7	0.01>p>0.005
28 day PCA	34 day C.R.	-0.35	7	n.s.

The correlation coefficient values (R) were derived from the conjunctival response (C.R.) and four hour PCA data described in Table 10.

D.F. : Degrees of freedom

n.s. : not significant

Table 12. Placental tansfer of conjunctival sensitivity

Guinea pig	Immunization	Maternal C.R.	Litter size	Offspring: positive C.R.
1.	Ovalbumin	2.0	2	$^{0}/_{2}$
2.	Ovalbumin	0.5	4	$^{4}/_{4}$
3.	Ovalbumin	2.0	3	$^{0}/_{3}$
4.	Ovalbumin	1.0	4	$^{3}/_{4}$
5.	Ovalbumin	2.0	4	$^{0}/_{4}$
6.	N.R.S.	0.5	4	$^{0}/_{4}$
7.	N.R.S.	2.0	2	$^{0}/_{4}$
8.	N.R.S.	1.0	3	$^{0}/_{4}$

500 g guinea pigs were immunized approximately six weeks before term with ovalbumin (500 µg intradermally) or normal rabbit serum (N.R.S.: 2 x 0.1 ml 20% dilution). Offspring were topically challenged within 24 hours of birth.

Maternal conjunctival challenge dose: 500 µg ovalbumin in 25 µl, or
2 drops N.R.S.

Offspring conjunctival challenge dose: 200 µg ovalbumin in 10 µl, or
1 drop N.R.S.

Conjunctival responses (C.R.) were scored visually, 30 minutes after challenge, in both cases.

2.1.4. Topical immunization with rabbit serum:

The fact that conjunctival challenge with an antigen can act as a stimulus to the immune system was demonstrated in an experiment in which 8 normal (unimmunized) guinea pigs were topically challenged with rabbit serum on a twice weekly basis in one eye only. At the sixth challenge after three weeks, two of the eight animals showed a positive conjunctival response, increasing to four of the eight guinea pigs after five weeks. In addition, positive contralateral eye challenge proved that systemic rather than local sensitivity had developed.

2.2. Serum antibodies in ovalbumin immunized guinea pigs.

A series of experiments were designed to further investigate the nature of the serum antibodies which result from intradermal ovalbumin immunization, and the effect of regular topical challenge thereon. A batch of 36 guinea pigs was divided into six groups and immunized with ovalbumin in the usual way. Groups I, III, and V were kept for 14, 28, and 56 days respectively without conjunctival challenge. Groups II, IV, and VI were kept for 28, 56 and 103 days, and topically challenged with ovalbumin 2, 5, and 8 times respectively. In addition, all 36 guinea pigs from the six groups were topically challenged immediately (30 minutes) prior to sacrifice, to establish final conjunctival sensitivity at death. Blood was collected from each guinea pig and sera prepared.

The 36 sera from groups I to VI were then examined for anti-ovalbumin antibody activity in the following tests: (1) haemagglutination, (2) haemolysis, (3) immunodiffusion, and (4) passive cutaneous anaphylaxis. The experimental design and overall results are summarised in Table 13.

No significant variation was recorded in conjunctival sensitivity between occasions in any of the regularly challenged groups (II, IV, and VI), or between conjunctival reaction scores in all six groups immediately prior to sacrifice, as shown in Table 13.

Table 13. Experimental design and results summary for Screen Tests with sera from guinea pig groups I to VI

Guinea pig Group	n	Sacrificed: days after immunization	Number of Topical Challenges with OVA: *	Latest Eye Challenge: mean ± s.e.m. **	T.O. S.R.B.C. agglutination: Log_2 units mean ± s.e.m.	T.O. S.R.B.C. Lysis: Titre	Ouchterlony Precipitin test: positive result	Four hour PCA TEST: mean diameter ± s.e.m. mm. 1:16 dilution:
I	5	14	0	1.6 ± 0.5	3.2 ± 1.5	<1:8	$^{0}/_{5}$	7.2 ± 2.6
II	6	28	2	2.2 ± 0.4	9.8 ± 0.9	<1:8	$^{3}/_{6}$	10.8 ± 2.6
III	6	28	0	1.5 ± 0.5	5.3 ± 1.5	<1:8	$^{1}/_{6}$	3.0 ± 1.9
IV	6	56	5	1.6 ± 0.4	10.5 ± 0.7	<1:8	$^{3}/_{6}$	11.7 ± 1.6
V	7	56	0	2.1 ± 0.4	9.4 ± 0.5	<1:8	$^{0}/_{7}$	5.1 ± 2.2
VI	6	103	8	2.0 ± 0.4	12.5 ± 0.6	<1:8	$^{6}/_{6}$	17.4 ± 1.2

Guinea pigs were divided into 6 groups, and immunized with ovalbumin by intradermal injection (500 µg). They were sacrificed at 14, 28, 56, or 103 days after immunization, and received topical conjunctival challenges with ovalbumin as shown (*). All guinea pigs, whether previously challenged or not, were topically challenged immediately (30 minutes) prior to sacrifice (**). The sera were tested *in vivo* by 4 hour passive cutaneous anaphylaxis (PCA), and *in vitro* by agglutination and lysis of tanned, ovalbumin coated, sheep red blood cells (T.O. S.R.B.C.), and by Ouchterlony precipitin test. Agglutination titres are expressed as Log_2 units. PCA scores refer to the mean ± s.e.m. results for 1:16 dilution at 4 hours (activites at 8 days were negative in all cases).

2.2.1. Haemagglutination:

The ability of doubling dilutions of each serum to agglutinate tanned sheep red blood cells coated with ovalbumin was assessed in MRC pattern agglutination plates. All titres were determined in duplicate with controls as specified in Materials and Methods.

The agglutination titres of sera in groups I, III, and V (guinea pigs unchallenged except immediately prior to sacrifice) showed a progressive increase (mean titres: 3.2, 5.3, and 9.4 respectively). This implied that the activity present at 14 and 28 days is part of an ongoing antibody synthesis possibly persisting for at least 56 days after immunization (Table 13). The mean titres for sera in groups II, IV, and VI (9.8, 10.5, and 12.5 respectively), were all higher than those for groups I, III, and V, and increased progressively with both time, and regular topical antigen challenge.

There was a statistically significant difference ($0.05>p>0.025$) between titres for groups II and III (challenged vs unchallenged at 28 days), but not between those for groups IV and V (challenged vs unchallenged at 56 days). Agglutination titres in group VI (multichallenged guinea pigs) were significantly higher than those observed in any other group ($p<0.001$). The mean titre for this group of 12.5 ($\log_2$ units) represented positive agglutination reactions at 1:8192 or 1:16384 serum dilutions.

Control treatments using tanned uncoated sheep red cells, nil serum (saline), and normal guinea pig serum (unimmunized animals) were all negative, ruling out the possibility of non-specific agglutination.

Therefore, guinea pigs immunized intradermally with ovalbumin for conjunctival challenge produced serum antibodies capable of agglutinating coated sheep red cells. Topical antigen challenge in the period 14-21 days after immunization significantly increased 28 day but not 56 day

titres. Regular challenge between 4 and 10 weeks after immunization further increased the antibody titres to higher levels than those found either at 28 or 56 days in groups II to V.

2.2.2. Haemolysis:

Sera from each group were tested for haemolytic activity against ovalbumin coated sheep red cells in MRC plates. In contrast to the high agglutination titres recorded earlier for many of the sera, all 36 sera failed to show any haemolytic activity, even at the lowest dilution tested (1:8). The test system itself was not at fault, as positive control sera used on each occasion gave high lytic antibody titres as expected.

2.2.3. Immunodiffusion:

Sera were tested for the presence of precipitating antibodies using double diffusion in agar gel plates. Each serum sample was placed in the centre well and diffused against a range of ovalbumin solutions (10 µg, 100 µg, 1.0 mg, and 10 mg per ml), to ensure adequate but not excess of antigen, as the latter resolubilizes precipitin complexes. The results were recorded non-quantitatively as a positive or negative precipitin reaction after incubation for 48 hours at 4°C.

With the sera from groups I, III, and V, only one serum sample from group III (animal 17) gave a positive precipitin result. In contrast, 3 of the six sera in each of groups II and IV, and all in group VI were positive (Table 13). It was therefore concluded that regular conjunctival antigen challenge induced the formation of precipitating serum antibodies to ovalbumin, which were either absent or undetectable in immunized unchallenged guinea pigs.

2.2.4. Passive cutaneous anaphylaxis:

Three fourfold dilutions of each serum sample were tested in duplicate for activity at 4 hour and 8 day latent periods of sensitization. The group mean reaction diameters (mm) shown in Table 13 and Figure 14 were calculated from the duplicate means for each individual serum sample.

Long term (8 day) activity of the test sera was either poor or zero. The short term (4 hour) activity was strong however, especially in those sera from regularly challenged guinea pigs. There were no significant differences between PCA scores for groups I, III, and V (unchallenged at 14, 28, and 56 days). The three challenged groups II, IV, and VI again showed a progressive increase in 4 hour PCA activity (group mean reaction diameters of 10.8, 11.7, and 17.4 respectively at 1:16 dilution).

The difference in 4 hour PCA activity between groups II and III (challenged vs unchallenged at 28 days) and groups IV and V (challenged vs unchallenged at 56 days) was statistically significant in both cases ($0.05>p>0.025$). The PCA scores in group VI (regularly challenged) were significantly higher than those for any other group ($p<0.001$). Two samples of normal guinea pig serum at 1:4 dilution used as zero controls both gave negative reactions.

2.2.5. Correlation coefficient analysis:

In addition to the previously mentioned statistically significant differences observed between sera from groups I to VI in the haemagglutination and PCA experiments, correlation coefficient analysis was performed on the data to check for positive correlation between individual serum antibody activities in each test and conjunctival sensitivity

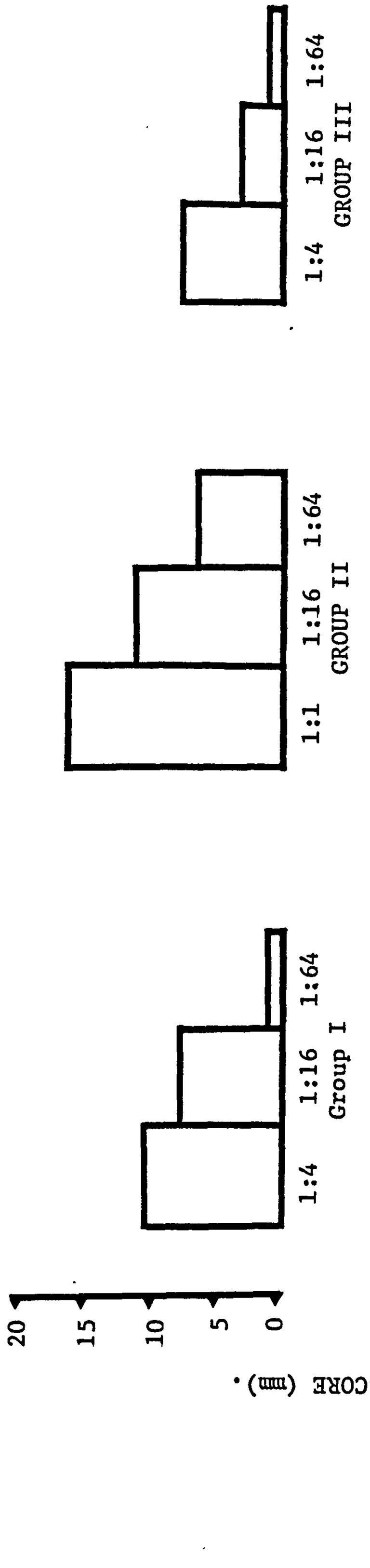


FIGURE 14. Passive cutaneous anaphylaxis activities of sera from guinea pig groups I to VI at three at 4 hour latent periods of sensitization.

The PCA activities were determined for each guinea pig serum sample in groups I to VI at three fourfold dilutions. The results, expressed above, were derived from the duplicate mean each individual serum sample.

(Table 14).

No positive correlation was observed between either PCA or haemagglutination scores and conjunctival sensitivity. However, there proved to be a highly significant correlation between the 4 hour PCA and haemagglutination activities themselves (Table 14).

2.2.6. Summary:

The results from three of the four tests employed (PCA, haemagglutination and immunodiffusion) re-emphasised the fact that regular conjunctival challenge is a continuing antigenic stimulus to the immune system. It results in increased short term PCA and haemagglutinating activity, and the appearance of precipitating antibodies. No complement fixing lytic antibody activity was detected in any of the sera from either immunized alone or regularly challenged guinea pigs. The positive correlation observed between short term PCA and agglutinating antibody activities indicated that these might be due either to the same serum antibody, or to different antibody populations (e.g. IgG antibody subclasses) synthesised quantitatively in parallel.

2.3. PCA time course studies with pooled sera from guinea pig groups I to VI : The Effect of Heat and 2-mercaptoethanol Treatment.

In view of the contrasting strong short term and poor long term mast cell sensitizing antibody activities observed in PCA experiments with sera from ovalbumin sensitive guinea pigs, it was considered essential to study the PCA activities of these sera over a range of sensitization latent periods between 4 hours and 8 days. In addition, the effect of heat and 2-mercaptoethanol on PCA activity was determined at each of the six chosen latent periods: 4, 8, and 24 hours, 2, 4, and 8 days.

Table 14. Correlation coefficient analysis between the severity of conjunctival response and serum antibody activities.

Correlation test.

Variable 1.	Variable 2.	R	D.F.	P
C.R.	4 hour PCA	0.03	34	n.s.
C.R.	HA.	0.21	34	n.s.
H.A.	4 hour PCA	0.67	34	$p<0.001$

The data used for the correlation coefficient analysis shown in this table was obtained from guinea pigs 1-36 in groups I to VI. These guinea pigs were immunized with ovalbumin and topically challenged as described in the text (2.2. 1-4). Conjunctival response (C.R.), 4 hour passive cutaneous anaphylaxis (PCA) and haemagglutination (HA.) data is summarised in Table 13.

n.s. : not significant

Serum pools were prepared by combining 1.0 ml volumes of individual sera from within each of the guinea pig groups I to VI. The serum pools I to VI used in the PCA time course study therefore corresponded directly to the guinea pig groups I to VI described previously (Table 13). The serum pools I to VI were then diluted (1:4, 1:4, 1:4, 1:16, 1:4, and 1:64 respectively), according to the known short term PCA activities of their constituent sera (Figure 14).

Each serum pool dilution was divided into four equal aliquots and treated as follows:

(a) : normal pool as untreated control,

(b) : heat treated pool (56°C for 4 hours),

(c) : 2-mercaptoethanol treated pool (dialysed sequentially against 2-mercaptoethanol, iodoacetamide, and 4 changes of PBS),

(d) : 2-mercaptoethanol control pool (dialysed sequentially against saline, iodoacetamide, and 4 changes of PBS).

The PCA activities were determined in quadruplicate for each of the four treatments of the six serum pools, at the six specified sensitization latent periods. Each sample was coded and randomised so that the PCA test was performed blind. The PCA test guinea pigs received 6 sequential intradermal injections at the appropriate times comprising one complete pool time course on each flank. The results for each of the six serum pools are shown in Figures 15 and 16.

The same characteristic PCA activity time courses were observed for each of the six serum pools over the six sensitization latent periods. The previously determined strong 4 hour activities increased

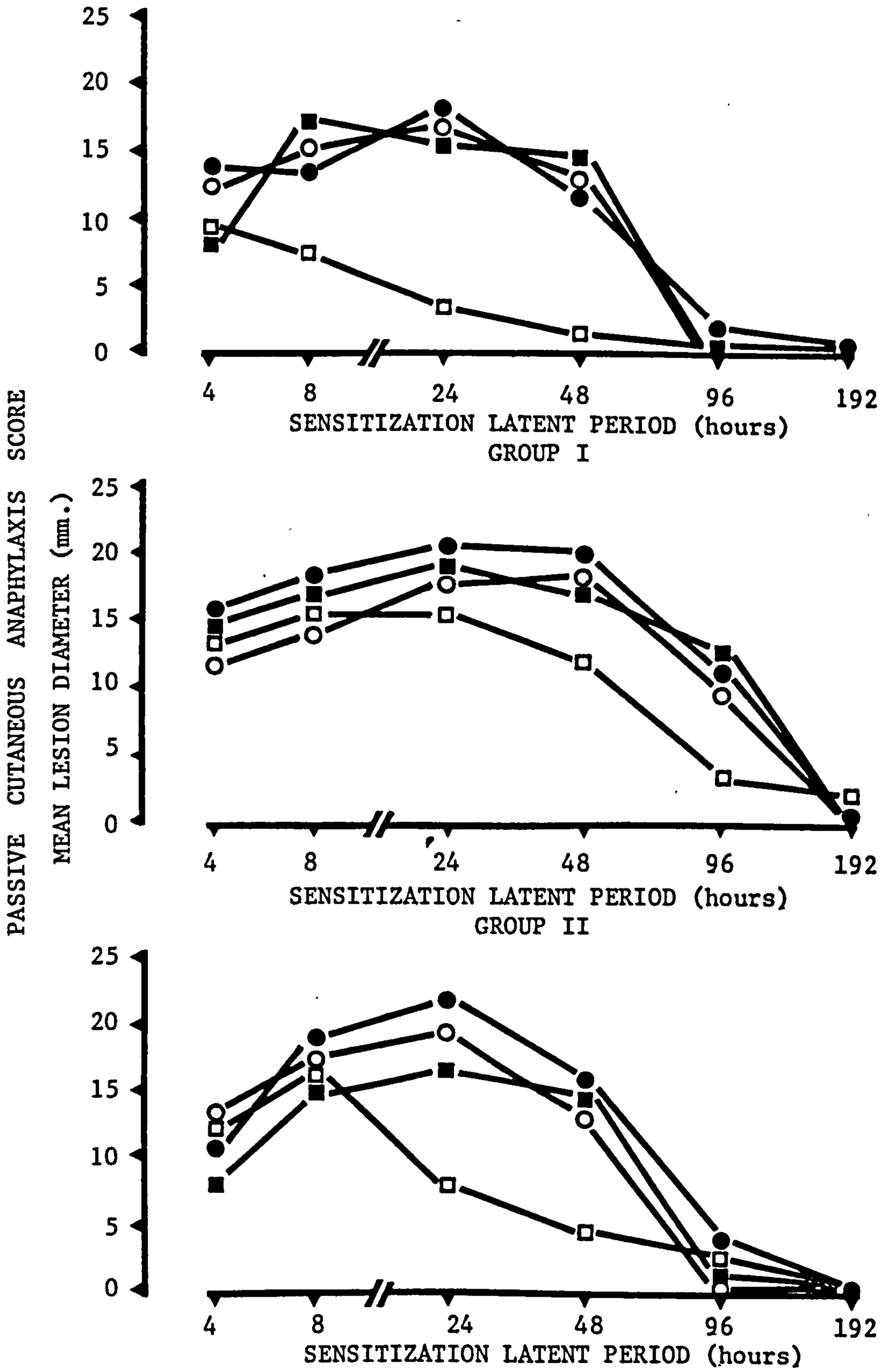


FIGURE 15. The effect of heat and 2-mercapto ethanol on pooled sera from groups I, II, and III.

Pools of serum from guinea pig groups I, II, and III (see Table 13) were tested for PCA activity at six latent periods of sensitization. Each serum pool was divided into four aliquots and tested as (i) untreated control (● - ●), (ii) heated at 56°C for 4 hours (o - o), (iii) iodoacetamide treated 2-mercaptoethanol control (■ - ■), and (iv) 2-mercaptoethanol dialysed (□ - □). PCA lesions were scored using the mean of two diameters measured at right angles. Each point is the mean of four determinations.

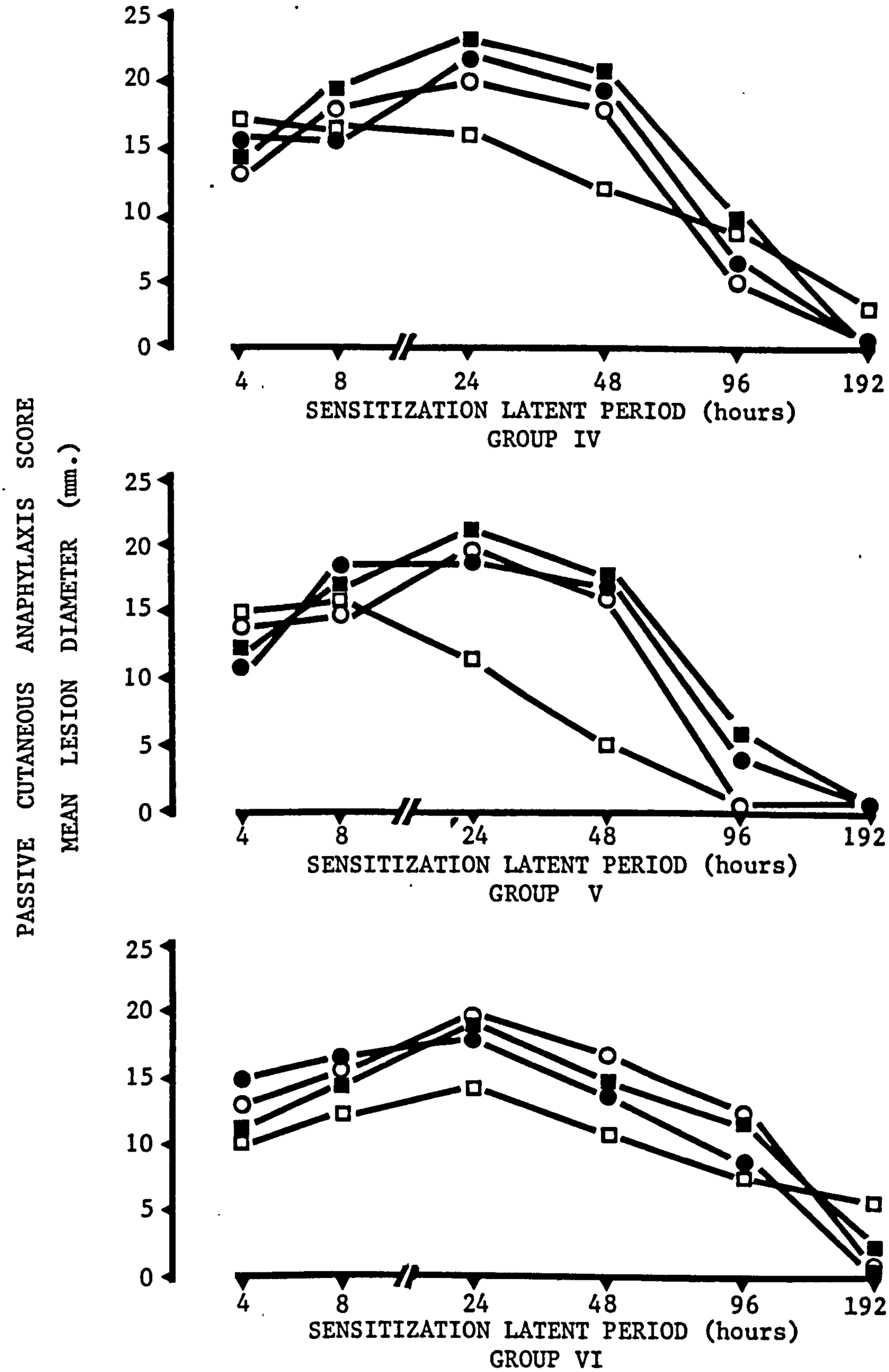


FIGURE 16. The effect of heat and 2-mercaptoethanol on pooled sera from groups IV, V, and VI.

Pools of serum from guinea pig groups IV, V, and VI (see Table 13) were tested for PCA activity at six latent periods of sensitization. Each serum pool was divided into four aliquots and tested as (i) untreated control (● - ●), (ii) heated at 56°C for 4 hours (o - o), (iii) iodoacetamide treated 2-mercaptoethanol control (■ - ■), and (iv) 2-mercaptoethanol dialyzed (□ - □). PCA lesions were scored using the mean of two diameters measured at right angles. Each point is the mean of four determinations.

at 8 hours, and were maximal at 24 hours in each pool. Thereafter, activity declined at 2 days (48 hours) and 4 days (96 hours), and was poor or absent after 8 days (192 hours). The PCA response curves for untreated and iodoacetamide alone treated (2-mercaptoethanol control) serum groups agreed closely in each case. In addition, no inhibition of PCA activity was evident following heat treatment in any of the serum pools.

A reduction in PCA activity occurred following 2-mercaptoethanol treatment in all six serum pools. PCA scores were generally similar to control values at 4 and 8 hour latent periods, but reduced at 24 and 48 hours. The inhibition was most marked with pools I, III, and V (pools of sera from unchallenged guinea pigs sacrificed at 14, 28, and 56 days respectively), but was also present in serum pools II, IV, and VI (sera from regularly challenged animals). Despite the small PCA treatment group sizes (4 determinations in each case), the inhibitory effect of 2-mercaptoethanol proved either marginally or highly significant (Student's t-test) with several of the serum pools at 24 and 48 hour sensitization latent periods (Table 15).

2.4. Separation of guinea pig 7S IgG_1 and IgG_2 antibodies.

A sample of pool VI guinea pig serum, known to contain high PCA and haemagglutinating activity, was chromatographed on Whatman DE-52 preswollen ion exchange cellulose and eluted on a continuous potassium phosphate buffer (pH 7.9) molarity gradient from 0.01 M to 0.3 M. The eluate protein absorption curve at 280 nm and the buffer molarity gradient across the column are shown in Figure 17. Four discrete elution peaks (three major and one minor) were observed before minimal protein elution was established after the passage of approximately 200 ml of

Table 15. 2-mercaptoethanol inhibition of serum pool PCA activities: statistical analysis.

Serum Pool	Latent period of sensitization	
	24 hours	48 hours
I	0.005>p>0.001	0.01>p>0.005
II	p>0.1	0.05>p>0.025
III	p>0.1	0.1>p>0.05
IV	0.1>p>0.05	0.05>p>0.025
V	p>0.1	0.05>p>0.025
VI	0.1>p>0.05	p>0.1

Serum pools I to VI correspond directly to guinea pig groups I to VI as shown in Table 13. Statistical analysis of results was by Student's t-test between control and 2-mercaptoethanol dialysed serum PCA activities at 24 and 48 hours. The results for 2-mercaptoethanol inhibition of pools III and V at 24 hours were not significant, due to small treatment group sizes and wide scatter of results.

$p>0.1$: not significant.

$0.1>p>0.05$: marginal significance.

$p<0.05$: activity significantly different from control score.

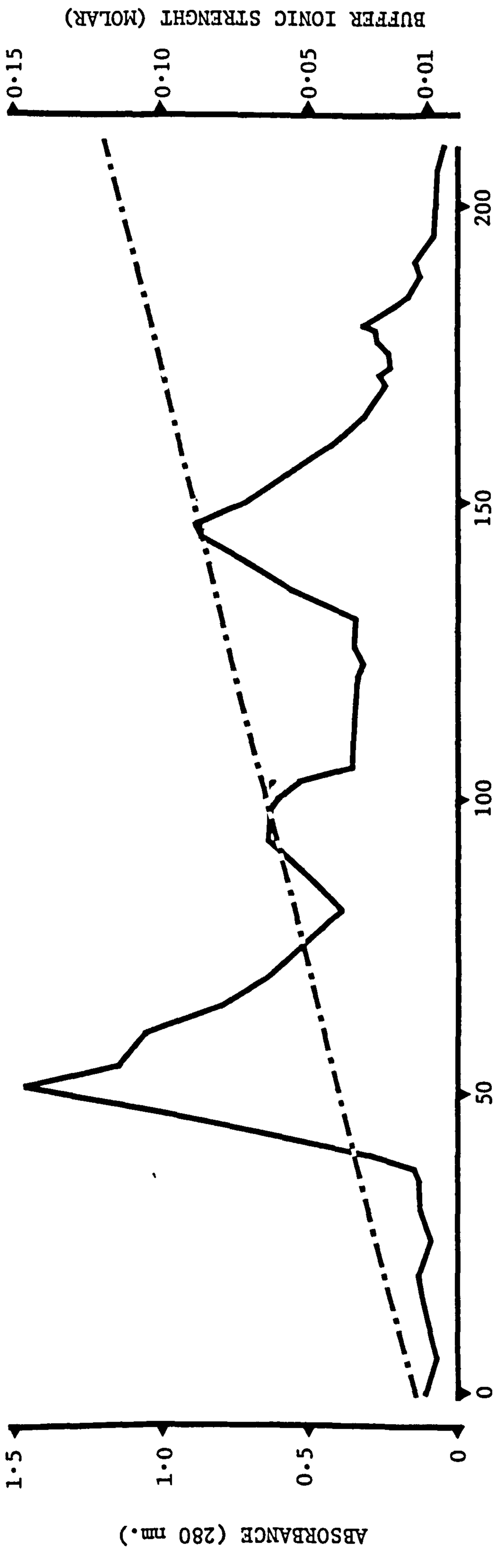


FIGURE 17. Protein elution curve for the fractionation of guinea pig serum on Whatman DE-52 ion exchange cellulose.

A sample of group VI pooled guinea pig serum (see Table 13) was eluted from a column of Whatman DE-52 ion exchange cellulose using a phosphate buffer molarity gradient between 0.01 m and 0.15 m (·—·—·). Absorbance of the eluate was monitored at 280 nm in individual 2.0 ml fractions.

buffer. The bulk of the serum proteins therefore eluted over the phosphate buffer molarity range 0.03 M to 0.1 M.

Individual 2.0 ml elution fractions were pooled into twelve major protein fractions and the volumes of each adjusted to 8.0 ml (equivalent to a 1:4 dilution of the column sample application volume). The twelve fractions were then tested for short term (4 hour) PCA and sheep red cell agglutinating activity by the previously described methods. The composition of the twelve major DE-52 fractions together with the test results are shown in Figure 18.

2.4.1. Haemagglutination:

Agglutinating activity was widely distributed over all twelve of the fractions. Fractions 2 to 6 proved the most active, with a gradual decline over fractions 7 to 12.

2.4.2. 4 hour PCA:

Fraction 1 (predominantly 7S IgG_2) showed minimal 4 hour PCA activity, as did fractions 9 to 12. The low activity observed in fraction 1 might have been due to slight contamination with IgG_1 antibody. Strong 4 hour PCA activity was present in fractions 2 to 6 (as for haemagglutination), being maximal in fraction 3.

These results collectively indicated at least a partial separation of 7S IgG_1 (gamma-1) and IgG_2 antibodies, with the former clearly exhibiting the bulk of the 4 hour PCA and haemagglutinating activity present (fractions 2 to 6).

2.4.3. The effect of 2-mercaptoethanol treatment:

The effect of dialysis against 2-mercaptoethanol on the PCA activities of the individual pool VI DE-52 ion exchange fractions 1-12

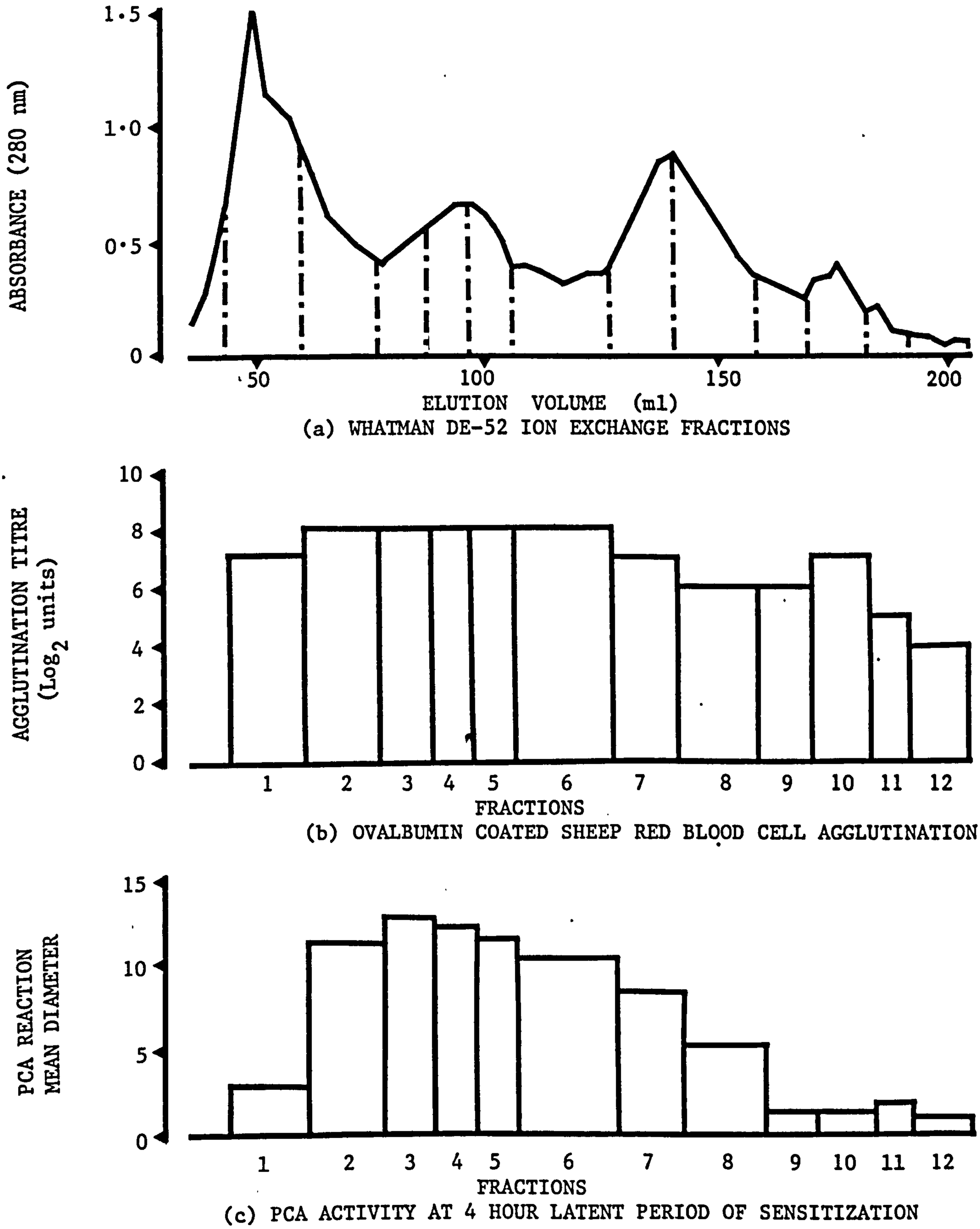


FIGURE 18. Agglutinating and PCA activity in Whatman DE-52 ion exchange chromatographed guinea pig serum.

A sample of pooled guinea pig serum (Group VI) was pooled and chromatographed on Whatman DE-52 ion exchange cellulose (see Figure 17). The eluate was pooled into 12 fractions as shown (a). Each fraction was tested in duplicate for agglutinating activity against ovalbumin coated sheep red blood cells (b), and for PCA activity using a 4 hour latent period of sensitization (c) where the result is the mean of four determinations.

was investigated in order to ascertain which of these fractions contained the 2-mercaptoethanol sensitive PCA antibody activity previously demonstrated in each of pools I to VI. The PCA activities of the resulting 24 DE-52 fraction-treatments (either 2-mercaptoethanol or saline dialysed) were determined in quadruplicate at the three sensitization time intervals (8, 24, and 48 hours), at which 2-mercaptoethanol inhibition had been previously observed (Figures 15 and 16). The results are shown in Figure 19.

Although short term (4 hour) PCA activity had been strongest in fraction 3 (Figure 18), control PCA activity at 8 hours was maximal in fraction 4, while 24 and 48 hours activities peaked in fraction 7. There was therefore a distinct shift in maximal PCA activity through fractions 3 to 7 at increasing latent periods of sensitization.

Significant 2-mercaptoetManol inhibition of this activity at 8 and 24 hours was only apparent in fractions 7 and 11 (Table 16). In contrast, marginal or highly significant inhibition was observed at the 48 hour latent period in fractions 2, 6, and 9-11. A decrease in PCA score was also recorded for fractions 7 and 8 which proved insignificant when analysed (Student's t-test), due to small treatment group sizes and large standard errors. Nevertheless, the overall picture suggested that the 2-mercaptoethanol stable PCA activity was largely present in fractions 2 to 5, while the 2-mercaptoethanol labile antibody activity occurred predominantly in the later fractions 6 to 11.

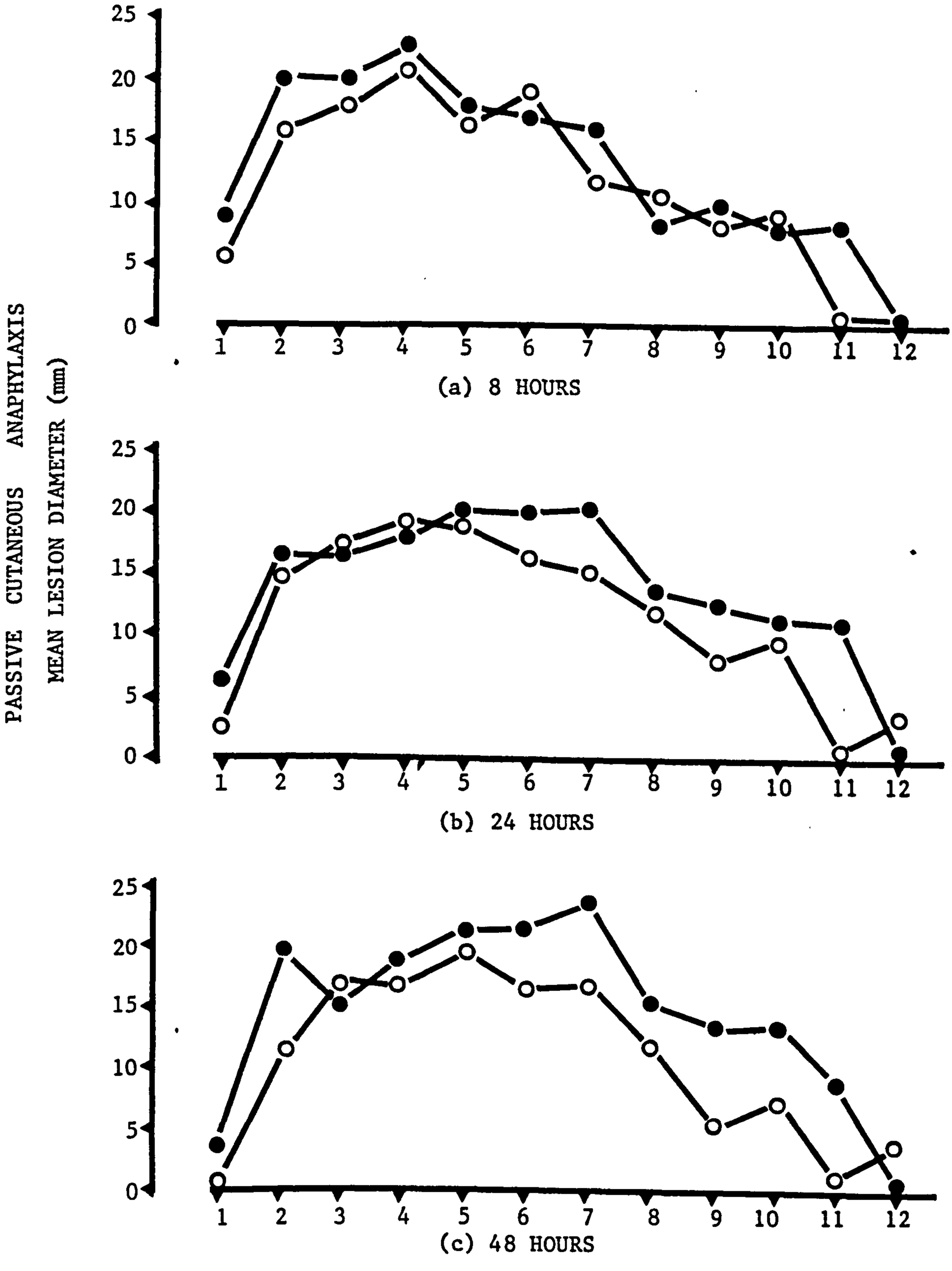


FIGURE 19. The effect of 2-mercaptoethanol on the PCA activities of DE-52 ion exchange chromatographed guinea pig serum.

Guinea pig serum (Group VI pool) was chromatographed on Whatman DE-52 ion exchange cellulose (shown in Figure 17). The eluate was pooled into 12 main fractions (see Figure 18). A sample of each fraction was then divided and treated by dialysis with 2-mercaptoethanol (**o** - **o**) or iodoacetamide alone (● - ●). The PCA activities of the 12 control (iodoacetamide) and 2-mercaptoethanol treated fraction samples were determined at 8 (a), 24 (b) and 48 (c) hour latent periods of sensitization.

Table 16. 2-mercaptoethanol inhibition of ion exchange fraction PCA activities from pool VI: statistical analysis.

DE-52 Fraction	Latent period of sensitization		
	8 hours	24 hours	48 hours
1	n.s.	n.s.	n.s.
2	n.s.	n.s	0.1>p>0.05
3	n.s.	n.s.	n.s.
4	n.s.	n.s.	n.s.
5	n.s.	n.s.	n.s.
6	n.s.	n.s.	0.005>p>0.001
7	0.01>p>0.005	0.05>p>0.025	n.s.
8	n.s.	n.s.	n.s.
9	n.s.	n.s.	0.025>p>0.02
10	n.s.	n.s.	0.05>p>0.025
11	0.025>p>0.01	0.005>p>0.001	0.05>p>0.025
12	n.s.	n.s.	n.s.

Fractions 1-12 correspond to the DE-52 fractions described in the text and Figure 18. Statistical analysis was by Student's t-test on PCA reaction scores determined in quadruplicate for each fraction at the three sensitization periods.

n.s. : not significant (p>0.1).

0.1>p>0.05 : marginal significance.

D I S C U S S I O N

The production of serum antibodies in the guinea pig, which are known to include IgM (macroglobulin), IgG_1 (gamma-1), IgG_2 (gamma-2), IgA, and IgE (Nussenzweig and Benacerraf, 1964; Nelson and Mildenhall, 1968; Pondman, Den Harink, and Van Es, 1969; Dobson, Rockey, and Soulsby, 1971; Dobson, Morseth, and Soulsby, 1971; Margni and Hajos, 1973a), will clearly be influenced by the type and dose of antigen used, the route of injection, and the adjuvant employed, if any (Parish, 1970c; Wong and Barbaro, 1976).

The electrophoretically slow IgG_2 antibody is formed in largest amounts when guinea pigs are immunized with antigens emulsified in complete adjuvant, whereas immunization with protein antigens in saline, as in the present study, results in a predominantly IgG_1 antibody response (Bencerraf, Ovary, Bloch, and Franklin, 1963). Serum IgE levels appear to be enhanced by the use of alumina gel adjuvants, infection with parasitic organisms, or simultaneous injection of antigen with Bordetella pertussis (Catty, 1969; Mota and Perini, 1970; Levine, Chang, and Vaz, 1971; Perini and Mota, 1972; Catty and Fraser, 1972; Ovary and Warner, 1972). Hicks, Opako, and Leach (1968) have observed the importance not only of the antigen immunization dose, but the period between immunization and challenge, finding that low antigen doses resulted in earlier maximal immediate hypersensitivity responses.

When investigating actively induced immediate hypersensitivity states in the guinea pig, most workers adopt and adhere to their own successsful immunization schedules, frequently making difficult a direct comparison of results. Nevertheless, evidence has accumulated

from the present and other studies to suggest the existence of three homocytotropic antibodies in the guinea pig, comprising (1) a short term sensitizing heat and 2-mercaptoethanol stable IgG_1, (2) a medium term sensitizing heat stable, 2-mercaptoethanol labile IgG_1, and (3) a reaginic (IgE) heat and 2-mercaptoethanol labile long term sensitizing antibody. The *in vivo* and *in vitro* activities of these homocytotropic antibodies are summarised and compared with those of the electrophoretically slow IgG_2 antibody in Table 17.

The partial separability of the two IgG_1 antibody populations on DEAE cellulose ion exchange resin observed in the present experiments has been previously reported by Perini and Mota (1972). Partial separability has also been reported using electrophoresis, where the 2-4 day sensitizing IgG_1 possessed slightly faster mobility in the most anodal region (Ovary and Warner, 1972). A clean separation of the two IgG_1 antibody populations by these or other methods has therefore yet to be achieved.

Successful purification of guinea pig IgE using ion exchange chromatography was first reported by Margni and Hajos (1973a, 1973b). These authors pointed out that the failure to isolate IgE by this method during previous investigations might be due to the use of stepwise rather than continuous molarity gradient techniques, at only 0.01 to 0.1M buffer strengths, whereas IgE eluted at 0.13M in their experiments. Ovary, Kaplan, and Kojima (1976) however, claim to have eluted heat-labile PCA activity from guinea pig serum between 0.035 and 0.05M using DEAE cellulose chromatography. In the present study, weak short and medium term sensitizing IgG_1 homocytotropic antibody activity was detected in the 0.12-0.15M eluate (Figure 19, fraction 11), but no

Table 17. A Summary of the Physicochemical and Biological Properties Guinea Pig Homocytotropic Antibodies.

	Homocytotropic Antibodies			
	IgG_2	IgG_{1a}	IgG_{1b}	IgE
Electrophoretic Mobility :	slow γ_2	fast γ_1	fast γ_1	β
Sedimentation Coefficient :	6.5 S	6.5 S	6.5 S	8-11 S
Ability to Fix Complement :	+	-	-	-
Haemagglutinating Activity :	+	+	+	-
Precipitin Activity :	+	+	+	-
Placental Transfer :	+	+	+	-
Optimum PCA latent period :	-	4-6 hours	2-4 days	7-8 days
Heat Lability :	-	-	-	+
2-mercaptoethanol Lability :	-	-	+	+

Data compiled from: Benacerraf et al., (1963); Reisfield and Hyslop (1966); Mota and Perini (1970, 1972); Dobson, Morseth and Soulsby (1971); Parish (1970); Dobson, Rockey and Soulsby (1971); Catty and Fraser (1972); Ovary and Warner (1972); Margni and Hajos (1973a, 1973b); Ovary, Kaplan and Kojima (1976).

IgE was present.

Although a PCA test will detect very low serum levels of homocytotropic antibody, and has been widely employed for this purpose, its inherent weaknesses must be recognised. These include stress induced by poor animal handling and bad injection technique, both of which can cause poor or diffuse cutaneous reactions. Dye recovery experiments have also shown discrepancies between the amount of antibody injected intradermally, and the quantity of dye subsequently recovered from skin reaction sites (Blum and Ovary, 1974). The use of precise methods for measuring tissue dye extrusion has therefore been reported to provide little additional information (Ovary, 1964; Blum and Ovary, 1974), and the measurement at specified serum dilutions of either antibody titres or comparative reaction diameters appears sufficient in practice.

The PCA experiments described in the text indicated the presence of both short and medium term sensitizing IgG_1 antibody populations in sera taken from guinea pigs immunized with ovalbumin for conjunctival challenge. PCA activity was 2-mercaptoethanol stable at 4 and 8 hours, but significantly inhibited at 24 and 48 hour latent periods. Activity at all these latent periods increased markedly in those sera from regularly challenged guinea pigs. PCA scores for sera from both challenged and unchallenged guinea pigs were low or nil at 4 and 8 day sensitization latent periods, indicating low or undetectable serum IgE levels at 14, 28, or 56 days after ovalbumin immunization, with or without conjunctival challenge. In contrast, strong 7 day PCA activity was recorded in sera from rabbit serum sensitive guinea pigs during preliminary experiments (see Table 9), suggesting that higher serum IgE levels are obtained following whole serum antigen administration.

No positive correlation occurred in any experiment between serum PCA activity and conjunctival response, for either ovalbumin or rabbit serum antigens. However, the serum levels of homocytotropic antibody detected in PCA experiments, be they IgG_1 or IgE, can serve as little more than a tentative guide to the amount and type of tissue mast cell fixed antibody, on which the conjunctival sensitivity of actively immunized guinea pigs must ultimately depend. Although IgE antibody activity was weak or absent in sera 14, 28, or 56 days after ovalbumin immunization, recent reports have shown that guinea pig serum IgE levels are frequently high very early in the antibody response to antigen (11-13 days), and subsequently low or undetectable after 3-4 weeks (Mota and Perini, 1970; Taylor and Roitt, 1973; Margni and Hajos, 1973a).

Two other investigations using guinea pigs immunized with rabbit serum (Dwyer, Turk, and Darougar, 1974) and ovalbumin (Taylor and Roitt, 1973) for conjunctival anaphylaxis have also indicated the presence of up to three homocytotropic antibodies in sera from reactive animals. The work of Dwyer et al. (1974) further suggested that the heat and 2-mercaptoethanol stable IgG_1 antibody may also persist in PCA skin sites rather longer (up to 10 days) than found in the present and other studies.

The agglutinating, precipitating, and short term PCA activities of the 2-mercaptoethanol stable IgG_1 antibody are well documented (Table 17), and a high positive correlation was obtained ($p<0.001$) between haemagglutination and 4 hour PCA results in this investigation. The probability that both of the IgG_1 antibody sub-populations possess haemagglutinating activity was reinforced by the high titres recorded in this test for the DEAE fractions containing the 2-mercaptoethanol

labile IgG_1 (fractions 9-11), in addition to those which contained predominantly 2-mercaptoethanol stable IgG_1 (fractions 2-5) as shown in Figure 18.

The absence of haemolytic antibody activity in any of the sera tested agrees with previous reports that saline immunization with low doses of protein antigens produces little detectable IgG_2 and IgM serum antibody. It further demonstrated that haemolytic antibody activity does not arise as a result of regular topical conjunctival antigen challenge. The initially strong and subsequently increasing haemagglutination antibody titres recorded in each of guinea pig groups I to VI showed that the IgG_1 antibody response is ongoing either up to or beyond 56 days after immunization, and confirmed that regular antigen conjunctival challenge serves as a continuing stimulus to the antibody producing system.

Whichever of the homocytotropic antibodies are responsible, there is no question that guinea pigs, once immunized, remain conjunctivally sensitive to antigen with or without regular topical challenge for at least 3-4 months. Long term persistence of non-serum detectable tissue mast cell fixed IgE is one plausible explanation. However, for either of the IgG_1 homocytotropic antibodies to be at least in part responsible, the serum levels of each must presumably remain sufficiently high to maintain tissue bound levels over an extended period. In fact, two pieces of evidence actually contraindicate IgG_1 involvement. Firstly, although regular conjunctival challenge significantly increases IgG_1 serum PCA activity, overall conjunctival sensitivity remains unaltered. Secondly, the serum half life of guinea pig IgG_1 antibody is reported as being only 7.1 days (LeFever and Ishizaka, 1972), which would

presumably necessitate high initial serum levels and continuing antibody synthesis over an extended period to maintain sensitivity.

In conclusion, (1) no positive correlation was observed in any experiment between serum PCA activity and conjunctival sensitivity, (2) PCA activity increased in sera taken from regularly challenged guinea pigs, whereas conjunctival sensitivity did not, and (3) serum detectable homocytotropic antibodies may not in any case accurately reflect the situation at tissue mast cell level. No definite conclusions can therefore be drawn concerning the precise nature of the anaphylactic antibodies responsible for immediate conjunctival hypersensitivity without further investigation.

CHAPTER THREE

PHARMACOLOGICAL CONTROL OF THE GUINEA PIG CONJUNCTIVAL RESPONSE TO ANTIGEN AND HISTAMINE

I N T R O D U C T I O N

The diverse pharmacological effects of histamine are now recognised as being mediated through two types of tissue receptor recently designated H_1 and H_2 (Ash and Schild, 1966). H_1 receptors have been shown to control peripheral vasodilation and the contraction of smooth muscle, and are antagonised by anti-histamines such as mepyramine or promethazine. In contrast, H_2 receptors stimulate gastric acid secretion, effect inotropic and chronotropic cardiac regulation, and are antagonised only by burimamide or metiamide (Black et al., 1972).

The potency of H_1 receptor antagonists in protecting guinea pigs against antigen induced bronchoconstriction is well documented (Armitage, Herxheimer, and Rosa, 1952; Collier and James, 1967; Bernauer, Hahn, and Giertz, 1969), although higher mepyramine doses are required to inhibit antigen induced bronchoconstriction than that due to histamine (Friebel, 1953; Bernauer, Hahn, and Giertz, 1969). H_1 receptor anti-histamines are also potent inhibitors of guinea pig PCA (Goose and Blair, 1969; Mielens, Ferguson, and Rosenberg, 1974), cutaneous inflammatory responses to thermal injury (Wilhelm and Mason, 1960), and antigen induced contraction of sensitized ileal and tracheal preparations *in vitro* (Joiner et al., 1974).

The H_1 receptor antagonist tested against the conjunctival response in the present study, triprolidine hydrochloride, is one of a series of phenylpyridlallylamines first noted for their anti-histamine activity by Adamson and Billinghurst in 1950. Its activity has subsequently been confirmed as equal to or greater than that of mepyramine in lung (Armitage, Herxheimer, and Rosa, 1952), skin (Movat et al., 1967), and ileum (Green, 1953).

Conjunctival sensitivity to 5-hydroxytryptamine (5-HT) in the guinea pig is poor, even at high topical or intraconjunctival doses (see Chapter One). In addition, other workers have suggested that 5-HT is probably not an important anaphylactic mediator in this species (Sanyal and West, 1958; Collier and James, 1967). Two 5-HT receptor antagonists, methysergide and B.W. 501C67, were used to further investigate the possible contribution of this agent to guinea pig conjunctival anaphylaxis. Both inhibit 5-HT induced rat paw oedema, and 5-HT stimulated contraction of rat uterus and guinea pig trachea (Green, unpb. obs.; Mawson and Whittington, 1970; Maling et al., 1974). Similarly, both inhibit reagin mediated PCA in the rat, but not guinea pig (Mielens, Ferguson, and Rosenberg, 1974; Follenfant and Follenfant, unpub. obs.). As a control for non-specific histamine receptor antagonism at high doses, the two compounds were also tested for activity against the histamine conjunctival response.

Sympathomimetic amines such as adrenaline and isoprenaline possess a recognised beneficial bronchodilator action in the treatment of asthma. However, a considerable weight of evidence has accumulated indicating an important additional effect of these agents at tissue mast cell level. Schild (1936) was the first to demonstrate that high concentrations of adenaline inhibit the antigen induced release of histamine from guinea pig lung. More recent investigations have shown that drugs capable of increasing intracellular levels of adenosine 3', 5'-cyclic phosphate (cAMP), either by adenyl cyclase stimulation (e.g. β-adrenergic agents), or cAMP phosphodiesterase inhibition (e.g. methyl-xanthines), are effective inhibitors of histamine release from passively sensitized human lung (Assem and Schild, 1969; Kaliner et al., 1971), human leucocytes (Lichtenstein and Margolis, 1968), guinea pig lung

(Assem, Pickup, and Schild, 1970), and rat peritoneal mast cells (Koopman, Orange, and Austen, 1970; Assem and Richter, 1971; Johnson, Moran, and Mayer, 1974).

Inhibition of mast cell mediator release by β-adrenergic agents can be prevented by β-adrenoreceptor blockade (propranolol) but not α-adrenoreceptor blockade (phentolamine). In contrast, α-adrenoreceptor agonists have been shown to increase intracellular levels of guanosine 3', 5'-cyclic phosphate (cGMP), and thus to enhance mediator release (Lichtenstein and De Bernado, 1971; Kaliner, Orange, and Austen, 1972; Tauber et al., 1973). Sympathomimetic amines also appear to reduce the histamine formation capacity via histidine decarboxylase of mast cells *in vitro* (Assem and Feigenbaum, 1972).

Salbutamol, the β-adrenergic agent chosen in the present study, is an active inhibitor of both IgG_1 and IgE mediated PCA reactions in guinea pigs (Martin, 1971; Mielens, Ferguson, and Rosenburg, 1974), and of histamine release from either passively sensitized human lung (Assem and Schild, 1969) or leucocytes (Perper, Sanda, and Lichtenstein, 1972). Although less potent than isoprenaline in most test systems, salbutamol is also reported to possess greater resistance to degradation by catechol-O-methyl transferase *in vivo* (Assem and Schild, 1969; Perper, Sanda, and Lichtenstein, 1972).

Clinical trials have conclusively proved the efficacy of disodium cromoglycate (DSCG) in the prophylaxis of bronchial asthma, allergic rhinitis, and allergic conjunctivitis (Howell and Altounyan, 1967; Morrison-Smith, 1968; Pepys et al., 1968; Robertson et al., 1969; Cohan et al., 1976). DSCG has no intrinsic bronchodilator, anti-histamine, or anti-inflammatory activity (Cox, 1967; Cox et al., 1970; Brogden,

Speight, and Avery, 1974), and is thought to act via an inhibitory effect on anaphylactic mediator release at mast cell level (Cox, 1971). Inhibition of immediate hypersensitivity reactions by DSCG has recently been reviewed by Cox (1976). This compound has shown high inhibitory activity in most reagin mediated systems, including rat and monkey PCA, and monkey or human bronchoconstriction (Altounyan, 1967; Goose and Blair, 1969; Orr, Gwilliam, and Cox, 1971).

Taylor and Roitt (1973) have previously investigated the effect of DSCG on conjunctival anaphylaxis in guinea pigs. DSCG was applied topically, simultaneously with antigen, over a specific period between 9 and 16 days after ovalbumin immunization. Inhibition of the conjunctival response was observed on days 9, 10, and 11. No inhibition was recorded on days 12 and 16, while slight but significant potentiation occurred on days 13 and 14.

The discovery of DSCG was unquestionably an important advance in the treatment of bronchial asthma and hay fever. However, being ineffective orally, it must be regularly applied topically, or be inhaled in a micronised powder form. A number of investigations have been undertaken in an attempt to develop a similarly active but orally effective agent. Doxantrazole is the result of one such investigation. This drug inhibits IgE mediated PCA and lung bronchoconstriction in rats, antigen induced histamine release from human chopped lung, and histamine release from passively sensitized rat peritoneal mast cells (Batchelor et al., 1975). Single oral doses also increase forced expiratory volume and peak expiratory flow rate in bronchial asthmatics (Haydu, Bradley, and Hughes, 1975).

Inhibition of intracellular cAMP specific phosphodiesterase (PDE)

has been proposed as a potential mechanism of action for anti-allergic drugs including DSCG and doxantrazole (Roy and Warren, 1974; Taylor et al., 1974a, 1974b; Tateson and Trist, 1976; Foreman and Garland, 1976). However, inhibition of cAMP PDE by anti-allergic drugs does not correlate with relative potency against mast cell histamine release *in vitro* for either DSCG, AH 7725, or theophylline (Varley and Skidmore, 1976). Furthermore, these and other anti-allergic agents of potential therapeutic value such as M&B 22948, ICI 74917, and BRL 10833, are more potent inhibitors of cGMP than cAMP hydrolysis *in vitro* (Bergstrand et al., 1977; Coulson et al., 1977). Considering that the KI cAMP/KI cGMP ratios for these compounds correlate with anti-allergic activity *in vivo* (rat PCA) and *in vitro* (human chopped lung), effects on intracellular mast cell cGMP levels, or on the cAMP/cGMP ratio, may be of equal or greater significance than effects on cAMP levels alone (Coulson et al., 1977). In the present study, DSCG and doxantrazole were compared as potential inhibitors of the antigen induced guinea pig conjunctival response when administered either topically, or by intravenous injection.

The view that prostaglandins (PG's) may function as chemical modulators of anaphylactic and inflammatory tissue reactions has gained considerable support in recent years. For example, $PGF_{2\alpha}$ has bronchoconstrictor activity in man (Mathé et al., 1973), while PGE's are bronchodilators (Cuthbert, 1969; Herxheimer and Roetscher, 1971; Smith and Cuthbert, 1972). Aspirin, an inhibitor of PG biosynthesis, has also been found to precipitate attacks of broncospasm in some asthmatics (Smith, 1972; Szczeklik et al., 1975). E and F series PG's may therefore be important in the maintenance of bronchial smooth muscle tone *in vivo*.

Histamine, SRS-A, and PG's are released from passively sensitized human and guinea pig chopped lung tissue following incubation with antigen (Piper and Vane, 1969; Piper and Walker, 1973). In addition, guinea pig lung releases a rabbit aorta contracting substance (RCS) originally believed to be a PG precursor (Piper and Vane, 1969; Palmer, Piper, and Vane, 1973; Gryglewski and Vane, 1971). RCS is now known to be a mixture of PG biosynthesis intermediates (the endoperoxides PGG_2 and PGH_2) and new mediators (thromboxanes A_2 and B_2), possessing short *in vivo* half lives (Kolata, 1975; Hamberg et al., 1976).

PG's are also released from guinea pig lung perfused with histamine, 5-HT, or SRS-A (Bakhle and Smith, 1972; Yen, Mathé, and Dugan, 1976; Engineer, Piper, and Sirois, 1977), and from guinea pig tracheal preparations superfused with histamine (Grodzinska, Panczenko, and Gryglewski, 1975). Furthermore, PG's themselves release histamine from human and guinea pig lung tissue, and from human or rat skin (Crunkhorn and Willis, 1971a, 1971b; Tauber et al., 1973; Hitchcock, 1974). PG's have been shown to inhibit *in vitro* histamine release from passively sensitized human lung (Tauber et al., 1973) and leucocytes (Lichtenstein et al., 1972), although only at concentrations sufficient to increase intracellular cAMP levels. Lower doses are reported to reduce tissue cAMP levels and enhance release (Tauber et al., 1973), while in human lung, indomethacin (a PG synthetase inhibitor) inhibits histamine release, and potentiates SRS-A release (Walker, 1973).

It is therefore unclear whether PG biosynthesis *in vivo* is a direct result of target mast cell activation, or a secondary effect following mediator release. Nevertheless, endogenous PG production

appears capable of regulating both the release and *in vivo* activity of other anaphylactic mediators (Walker, 1973). The effect of PG's E_1, E_2, and $F_{2\alpha}$ on the guinea pig conjunctival response was investigated by incorporating low PG doses into antigen and histamine intraconjunctival challenge solutions. The possible contribution of endogenous PG biosynthesis to the conjunctival response was studied by treating guinea pigs with the prostaglandin synthetase (cyclo-oxygenase) inhibitor indomethacin prior to challenge.

Experiments were also included in the present study to investigate the effects on guinea pig anaphylaxis of the anti-inflammatory steroid dexamethasone (Hicks, 1965; Kovacs, 1965), previously shown to be active in guinea pig tissues (Kurihara and Shibata, 1975), and the topically applied local aneasthetic lignocaine.

The latter agent was initially employed to alleviate any discomfort during and immediately following an intraconjunctival injection. Lignocaine was therefore tested for effects against the conjunctival response itself.

MATERIALS AND METHODS

Animals:

Outbred albino Dunkin-Hartley strain guinea pigs, approximately 300g at immunization, were purchased from Charles River U.K. Ltd., or from the Redfern Animal Breeders Ltd.

Immunization:

Ovalbumin (Koch-Light; 5x recrystallised) was given by intradermal injection (500 µg in buffered saline) as described in Chapter One.

Challenge:

Topical and intraconjunctival challenge was performed as described in Chapter One. Standard topical doses of ovalbumin (500 µg), histamine (250 µg), and compound 48/80 (7.5 mg) were used except where stated.

Reaction Assessment:

The visual (0 to 4+) scoring system was routinely employed, except following intraconjunctival injection challenge in guinea pigs pretreated with intravenous Evans Blue. In the latter case, either reflectometer values alone, or reflectometer difference value x upper bulbar/palpebral conjunctival width was used (see Chapter One).

Chemicals:

B.W. 501C67 (α-anilino-N-2-m-chlorophenoxypropylacetamidine: Wellcome Research Labs.).

Compound 48/80 (Wellcome Research Laboratories).

Dexamethasone (Decadron: Merck, Sharp and Dohme Ltd.).

Disodium cromoglycate (Intal: Fisons Ltd.).

Doxantrazole (Wellcome Research Laboratories).

Histamine dihydrochloride (Sigma Chemical Co.).

Indomethacin (Sigma Chemical Co.).

Methysergide hydrogen maleate (Sandoz).

Pentabarbitone sodium (Nembutal).

Prostaglandins E_1, E_2, and $F_{2\alpha}$ (Cambrian Chemicals Ltd.).

Salbutamol sulphate (Ventolin: Allen and Hanbury Ltd.).

Triprolidine hydrochloride (Actidil: Wellcome Research Labs.).

Lignocaine hydrochloride (Xylocaine: Astra Chemical Co.).

Experimental design:

Two alternative experimental designs may be used to obtain pharmacological control and test data during the investigation of conjunctival reaction antagonist activities. Both methods possess inherent advantages and disadvantages:

(1) Control and test conjunctival responses may be determined by using both eyes of the same individual guinea pigs. This type of experiment allows improved sensitivity control, and the use of smaller treatment group sizes. However, Home Office guidelines stipulate the avoidance of subjecting guinea pigs to simultaneous conjunctival reaction in both eyes if possible. This method therefore requires a 2-3 hour delay between challenges, to allow complete control reaction recovery before drug testing in the contralateral eye. However, it does also facilitate the removal from the experiment of poor or zero antigen responders at the control challenge stage.

(2) Challenge in one eye only of different guinea pigs assigned to either control or drug treatment test groups removes the problem of

a delay between challenges. One disadvantage of this method is that larger treatment groups are required to compensate for the variation in individual antigen sensitivity present in all batches of similarly immunized guinea pigs.

The second type of experimental design was most often used, with separate control and test groups of animals challenged in a random treatment order. All experiments were performed 'blind' at both the challenge and scoring stages, in order to eliminate any possible personal bias when using the visual reaction assessment system.

Statistical analysis of results:

The method of pharmacological data analysis chosen when using an arbitrary visual reaction scoring system, as in the present study, requires careful consideration. Principally, the choice lies between using either a parametric or non-parametric type of statistical test. The decision made may initially affect the significance of the results obtained, and accordingly the validity of any conclusions to be drawn.

Briefly, a parametric test, of which Student's t-test is probably the most widely used example, makes a number of important assumptions about the data to be analysed. Data sets are assumed to be derived from a normally distributed population, and consequently to possess similar variances. In addition, the results are generally derived from a clearly defined ordinal scale of known linearity (e.g. PCA reaction diameters, antibody titres, voltage electrode potentials, etc.). In contrast, a non-parametric test makes no such assumptions about the population to be analysed. The results are therefore valid for data populations of possibly differing variances, measured using either ordinal or nominal scales.

Clearly, the choice of test for the analysis of conjunctival response data must wholly depend on the type of reaction assessment employed. Measurement of dye extrusion into the conjunctiva with the reflectometer would permit the use of a parametric test. On the other hand, the visual scoring method, although on a numerical scale, is only a more sophisticated form of 'ranking' reaction severity. There is no clear evidence to suggest that the visual 0 to 4+ scoring scale is linear, and it may rather be logarithmic. Furthermore, it is also a scale possessing an artificially predefined upper limit (4+), the level of which may not be totally valid.

For these reasons, the visually scored results described in the text have been analysed using the non-parametric Wilcoxon Rank Sum Test rather than by parametric methods. In this test, both control and drug test scores are placed in overall rank order. The rankings of the test sample (N_1) are summed and compared with the value for the controls (N_2). By reference to the appropriate statistical tables the significance (p value) of the test group data is obtained for the appropriate treatment group sizes N_1, N_2. As in the Student's t-test, $p<0.05$ is taken as indicating a significant result (see Siegal, 1956).

For the same reasons as those which led to the adoption of the Wilcoxon Rank Sum Test (WRST), the visually scored conjunctival response data presented graphically in the text is expressed in terms of absolute reaction scores, rather than by % inhibition values.

R E S U L T S

3.1. Inhibition of the Conjunctival Response by Triprolidine.

The inhibitory activity of the H_1 receptor antagonist triprolidine against topical ovalbumin, histamine, and compound 48/80 induced conjunctival responses was investigated. The drug was administered either systemically (by intraperitoneal injection 30 minutes prior to challenge), or topically (incorporated into the respective challenge solutions).

Conjunctival responses obtained following systemic triprolidine pre-treatment doses are shown in Figure 20. Compound 48/80 reactions were significantly inhibited at all three dose levels tested ($p<0.01$). Histamine induced responses were also potently inhibited in a dose-related fashion ($p<0.01$). Total inhibition of the histamine conjunctival response was observed following a 3.0 mg/kg triprolidine dose in most guinea pigs. In contrast, inhibition of the antigen reaction was only partial, although significant at doses between 1 and 10 mg/kg. Inhibition was maximal at the 3.0 mg/kg dose level as in the case of histamine, but no additional suppression of the antigen response was observed at the higher 10 mg/kg dose.

Topical application of triprolidine simultaneously with challenge also resulted in a dose-related inhibition of both antigen and histamine induced conjunctival erythema and oedema (Figure 21). This effect was significant ($p<0.01$) at triprolidine doses between 2.5 and 250 µg. As when administered systemically, topical triprolidine was able to completely abolish the conjunctival response to histamine. However, the antigen reaction was again only partially inhibited, even with a

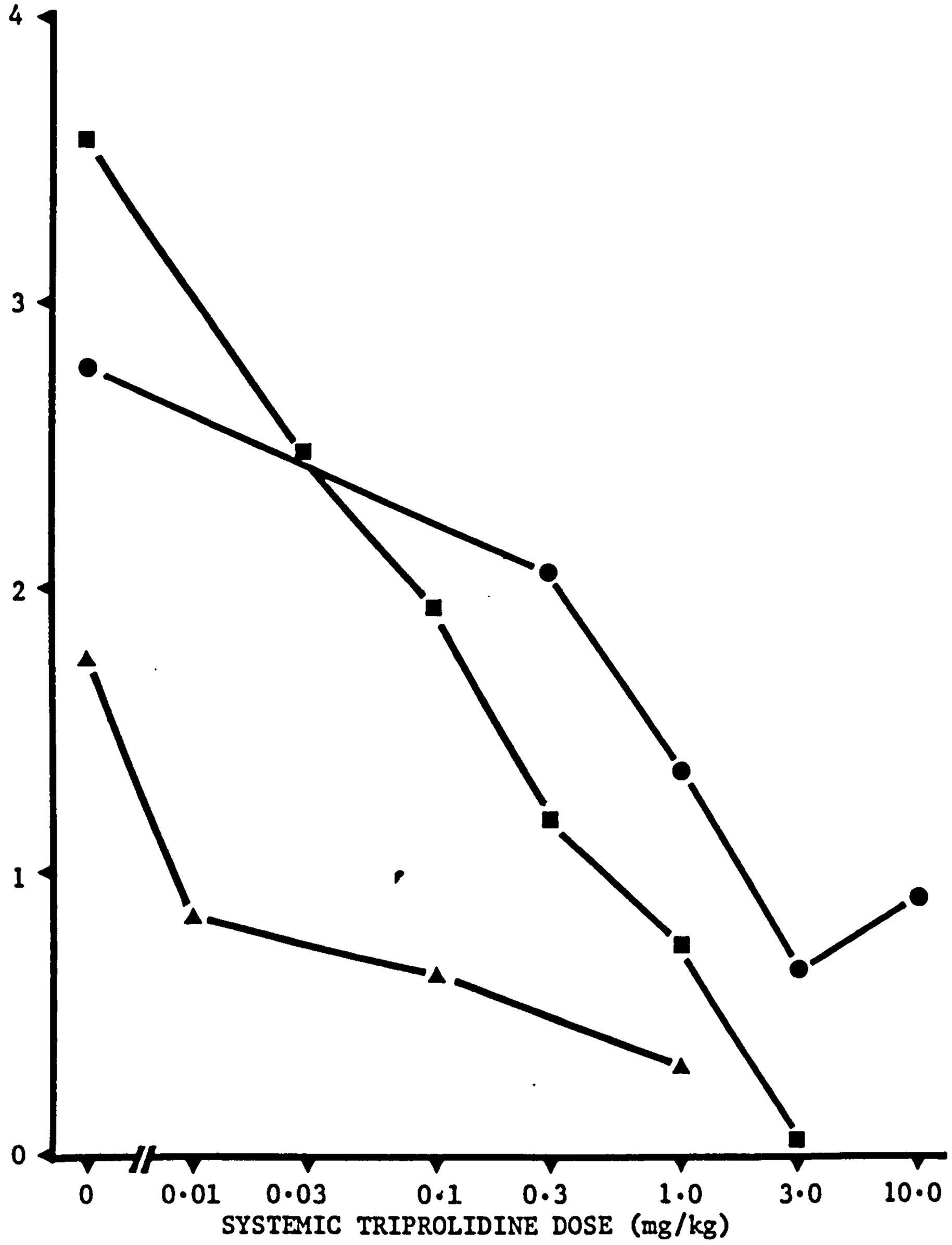


FIGURE 20. Inhibition of the guinea pig conjunctival response to ovalbumin (● - ●), histamine (■ - ■) and compound 48/80 (▲ - ▲) by systemic doses of triprolidine.

Triprolidine was given in saline by intraperitoneal injection 30 minutes prior to topical challenge. Control or drug treated guinea pigs were challenged in one eye only. Not less than 10 guinea pigs were challenged at each dose level in all three cases.

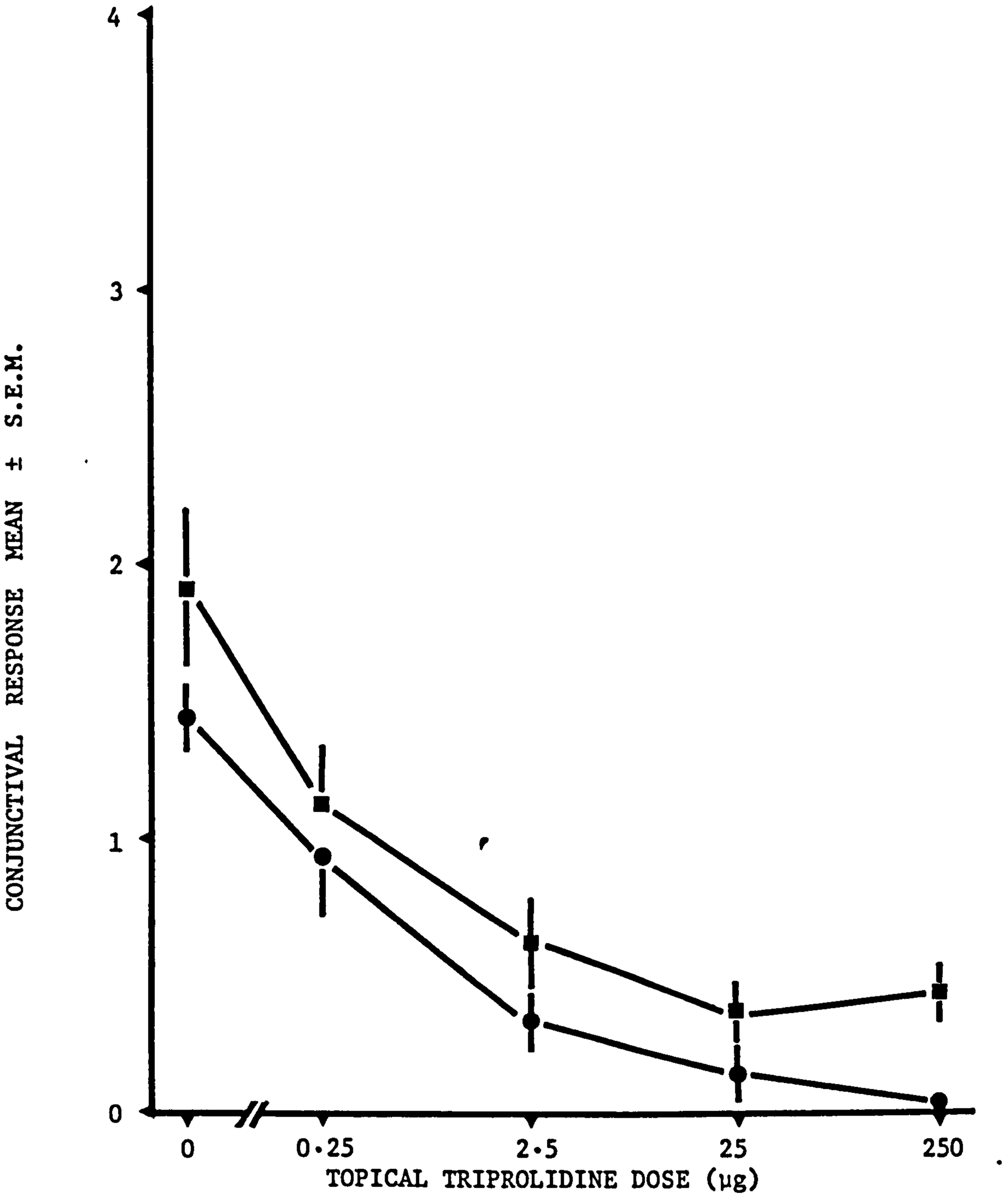


FIGURE 21. Inhibition of the conjunctival response to topical ovalbumin (● - ●) and histamine (■ - ■) by topically applied triprolidine.

Triprolidine at the doses stated was incorporated into the ovalbumin and histamine topical challenge solutions. Group sizes used were not less than 8 guinea pigs per treatment dose. Each guinea pig (control or drug treated) was challenged in one eye only.

tenfold increase in drug dose.

The evidence indicating a sequential release of anaphylactic mediators in immediate hypersensitivity reactions is now substantial (Brocklehurst, 1960; Kaliner, Wasserman, and Austen, 1973; Jones and Kay, 1974). An explosive release of histamine appears to be followed by a slower formation and progressive release of other active substances such as SRS-A and PG's. If this holds true for the guinea pig conjunctival response to antigen, then the effect of triprolidine might be expected to be greater during the histamine release phase in the early stages of the reactions.

To test the above hypothesis, conjunctival reactions were assessed in control and triprolidine treated (3.0 mg/kg) guinea pigs at 5 minute intervals from 5 to 30 minutes after ovalbumin challenge. The results (Figure 22) did not show increased triprolidine inhibitory activity in the early stages of the response (5-15 minutes), but rather a continuing and highly significant ($p<0.01$) suppression of reaction severity over the entire reaction time course. Accelerated recovery from the resultant milder responses was observed in the drug treated group.

3.2. The Effect of 5-HT Antagonists on the Conjunctival Reaction.

In order to investigate the contribution, if any, of 5-HT to guinea pig conjunctival anaphylaxis, the effects of two recognised 5-HT antagonists (B.W. 501C67 and methysergide) were studied. Both drugs were administered by intraperitoneal injection 30 minutes prior to challenge, and were tested against both ovalbumin and histamine responses (the latter to act as inhibition specificity controls).

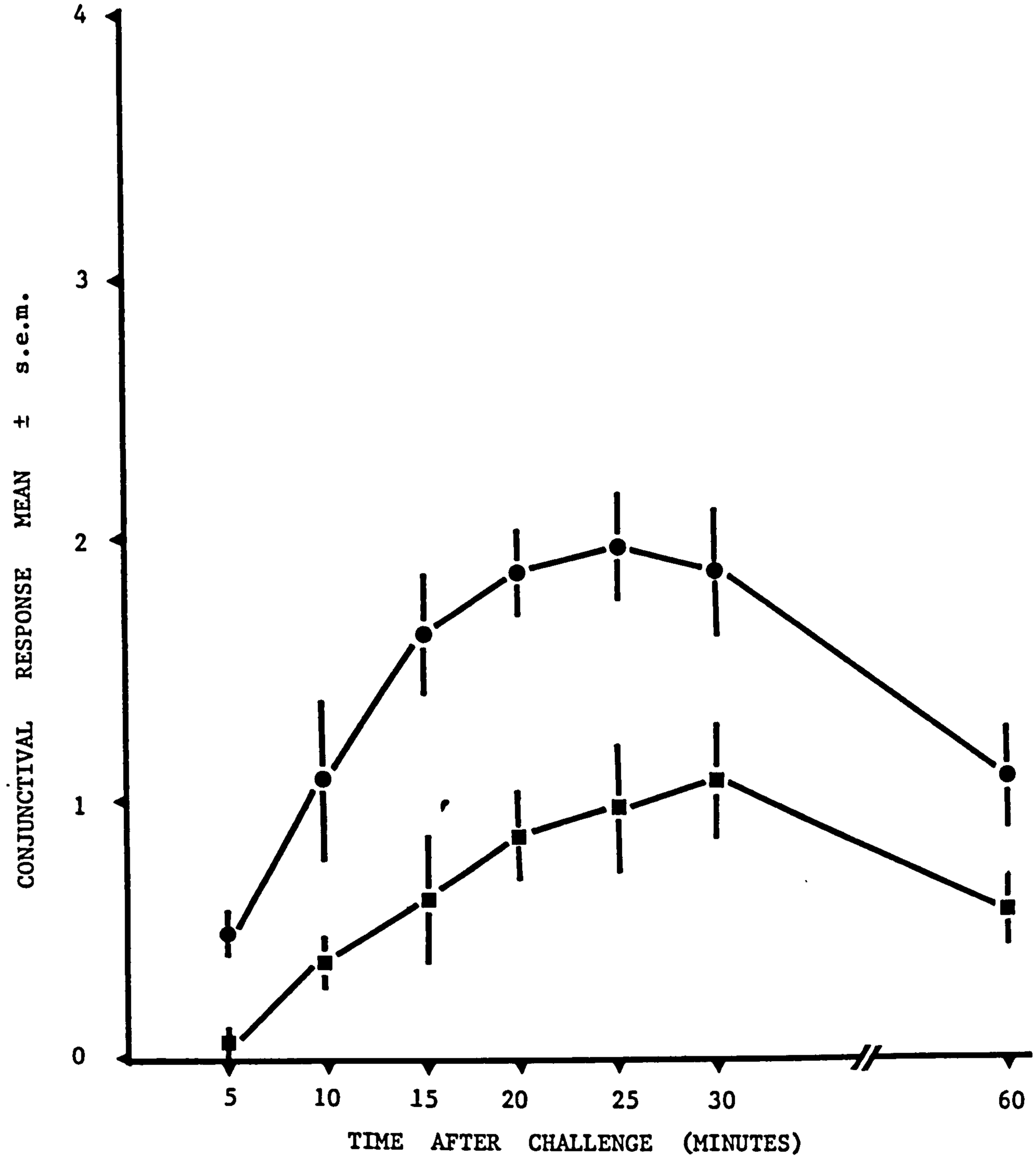


FIGURE 22. Inhibition of the conjunctival response to topical ovalbumin challenge by triprolidine at varying intervals after challenge.

Two groups of 12 guinea pigs were treated with saline (● - ●) or triprolidine (■ - ■ ; 3·0 mg/kg) by intraperitoneal injection 30 minutes prior to topical challenge with ovalbumin. Conjunctival responses were scored at 5 minute intervals up to 30 minutes after challenge, and again at 1 hour after challenge.

In two separate experiments, 3.0 mg/kg but not 0.3 mg/kg doses of B.W. 501C67 effected significant ($p<0.01$) inhibition of the ovalbumin challenge response (Table 18). Methysergide also produced a significant reduction in ovalbumin reaction severity ($p<0.05$) at both dose levels tested (1.0 and 10.0 mg/kg). However, the specificity of receptor antagonism for both drugs at the dose levels employed must remain in question. B.W. 501C67 (3.0 mg/kg) in one experiment, and methysergide (10.0 mg/kg) in another, also significantly inhibited the conjunctival response to histamine (Table 18).

3.3. Inhibitory Activities of two Anti-Allergic Agents: Disodium cromoglycate (DSCG) and Doxantrazole.

These two drugs were tested for inhibitory activity against the antigen conjunctival response by (1), topical application of drug simultaneously with antigen by incorporation into challenge solutions, and (2), intravenous (ear vein) injection of drug immediately prior to ovalbumin challenge. Guinea pigs were initially challenged at approximately 14 days after immunization, and were used for subsequent experiments at weekly intervals.

(1) The data which resulted from topical DSCG and doxantrazole administration is shown in Figure 23. Each inhibition curve was built up over a series of experiments using three separate batches of animals. Both drugs proved to be ineffective at the lowest topical dose tested (75 µg). However, a progressive dose-related inhibition was observed in each case for the three higher dose levels. This effect was significant for DSCG and doxantrazole (both $p<0.01$) following doses of 750 µg and 2.5 mg. Determination of

Table 18. Inhibition of the antigen and histamine induced conjunctival response with 5-hydroxytryptamine antagonists.

Challenge	Drug	Dose mg/kg	C.R. (mean ± s.e.m.) Control	Test	WRST
OVA	B.W. 501C67	0.3	2.3 ± 0.2	2.2 ± 0.3	n.s.
OVA	" 501C67	0.3	2.3 ± 0.3	1.8 ± 0.4	n.s.
OVA	" 501C67	3.0	2.3 ± 0.2	1.3 ± 0.2	p<0.01
OVA	" 501C67	3.0	2.3 ± 0.3	0.9 ± 0.2	p<0.01
OVA	METH.	1.0	2.3 ± 0.3	1.3 ± 0.3	p<0.05
OVA	METH.	10.0	2.3 ± 0.3	1.4 ± 0.2	p<0.05
HIS	B.W. 501C67	3.0	2.2 ± 0.2	2.0 ± 0 3	n.s.
HIS	" 501C67	3.0,	2.5 ± 0.3	1.4 ± 0.2	p<0.05
HIS	METH.	10.0	2.5 ± 0.3	1.3 ± 0.3	p<0.05

Methysergide (METH.) and B.W. 501C67 were given by intraperitoneal injection 30 minutes prior to topical challenge with ovalbumin (OVA) or histamine (HIS). The results are expressed as mean ± s.e.m. for visually scored conjunctival responses (C.R.), and were analysed using the Wilcoxon Rank Sum Test (WRST). All treatment group sizes were $\geq$ 6 guinea pigs.

n.s. : not significant

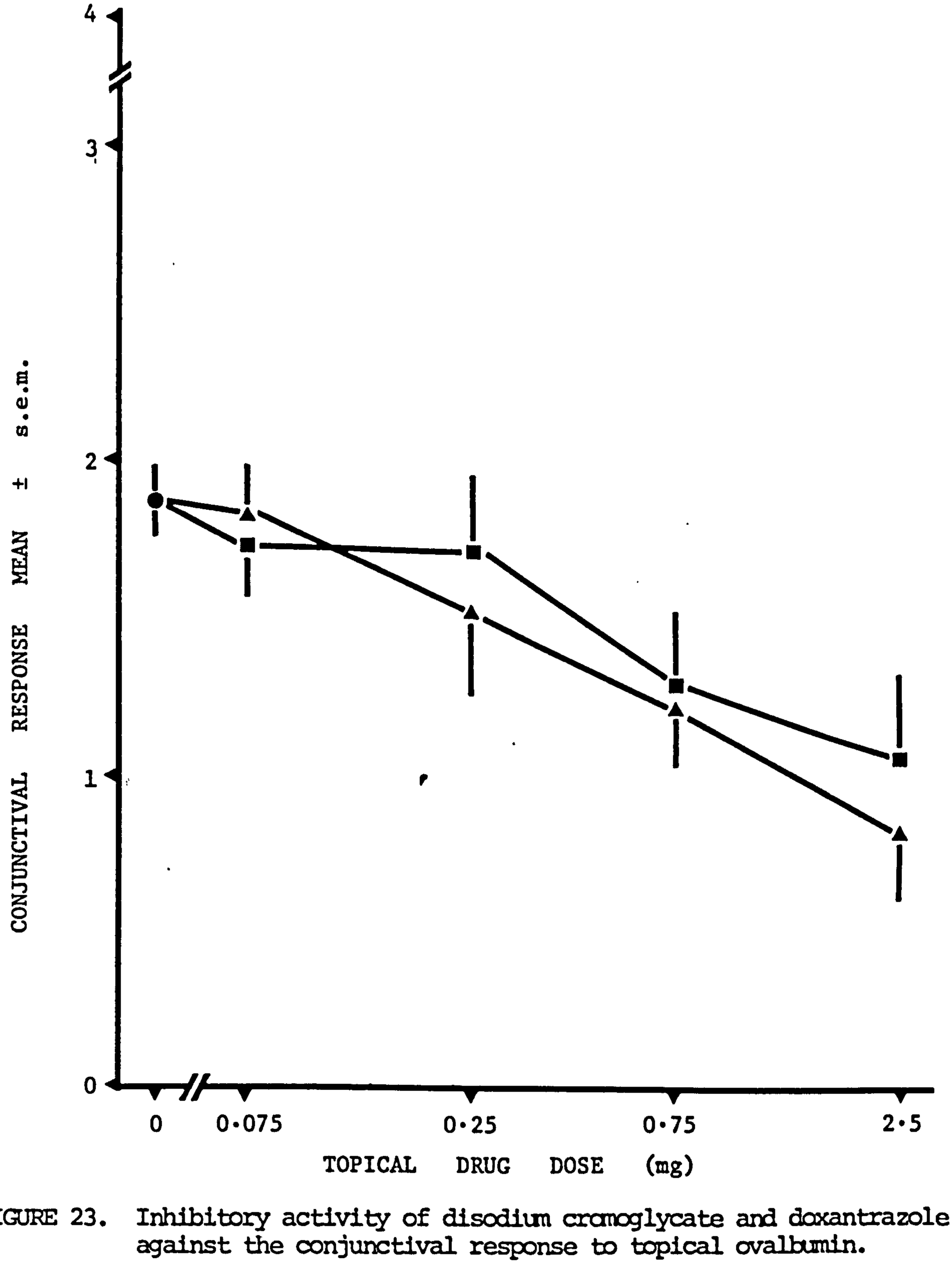


FIGURE 23. Inhibitory activity of disodium cromoglycate and doxantrazole against the conjunctival response to topical ovalbumin.

Disodium cromoglycate (■-■) and doxantrazole (▲-▲) were administered topically, incorporated in the ovalbumin challenge solution. The inhibition curves shown are comprised of data obtained from a series of six experiments with three separate batches of guinea pigs. Each guinea pig was challenged in one eye only. Total group sizes were > 20 guinea pigs for each treatment dose.

relative potency by performing linear regression analysis on the data showed that there was no significant difference between the topical inhibitory activities of the two compounds. In some experiments, evidence of minor occular irritation (blinking) was noted immediately after instillation of the highest topical doxantrazole doses.

(2) Intravenous pretreatment with DSCG (30 mg/kg) immediately prior to challenge produced no significant inhibition of the ovalbumin conjunctival response. In contrast, doxantrazole was again able to inhibit the ovalbumin response in dose-related fashion when given by this route (Figure 24). The effect was significant at the two highest doses ($p<0.01$).

Taylor and Roit (1973) have previously described the variable effects of DSCG on antigen induced conjunctival anaphylaxis in guinea pigs challenged over a specific period from 9 to 16 days after immunization. In an attempt to repeat these findings, separate groups of guinea pigs immunized and challenged according to my own protocols were used to investigate the inhibitory activity of DSCG at similar dose levels between 8 and 15 days after immunization. At the chosen dose of DSCG (250 µg) no significant inhibition or potentiation of the ovalbumin conjunctival response was observed on any of the days in this period (Figure 25).

As high doses of DSCG and doxantrazole were required to inhibit the antigen conjunctival reaction, a series of inhibition specificity control experiments were also performed by testing both of these drugs against the histamine conjunctival response. Three types of challenge system were used:

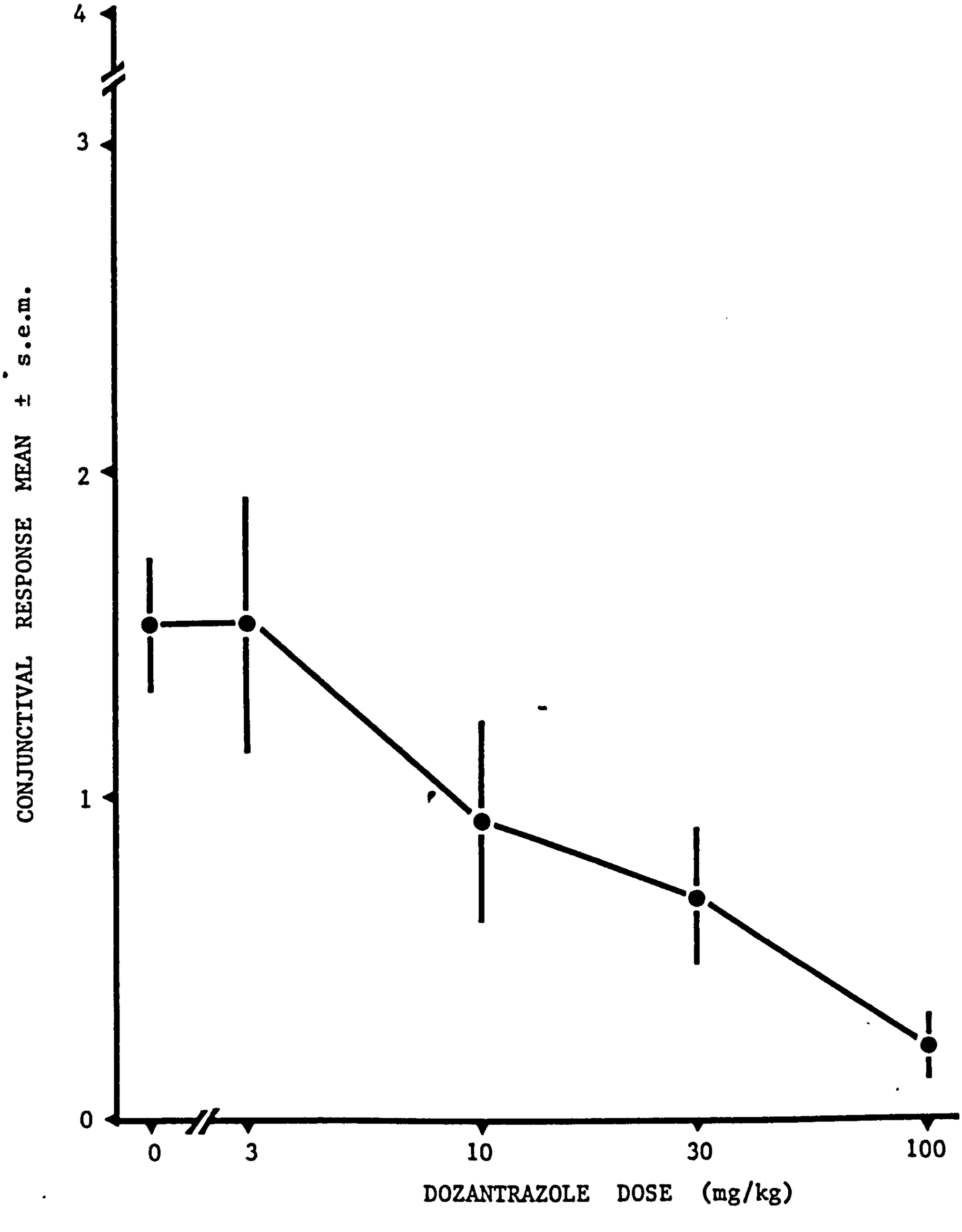


FIGURE 24. Inhibition of the conjunctival response to topical ovalbumin challenge by systemically administered doxantrazole.

Doxantrazole was given by intravenous injection immediately (2 minutes) prior to challenge. Group sizes were > 8 animals for each treatment dose. Disodium cromoglycate (30 mg/kg i.v.) was inactive in this test.

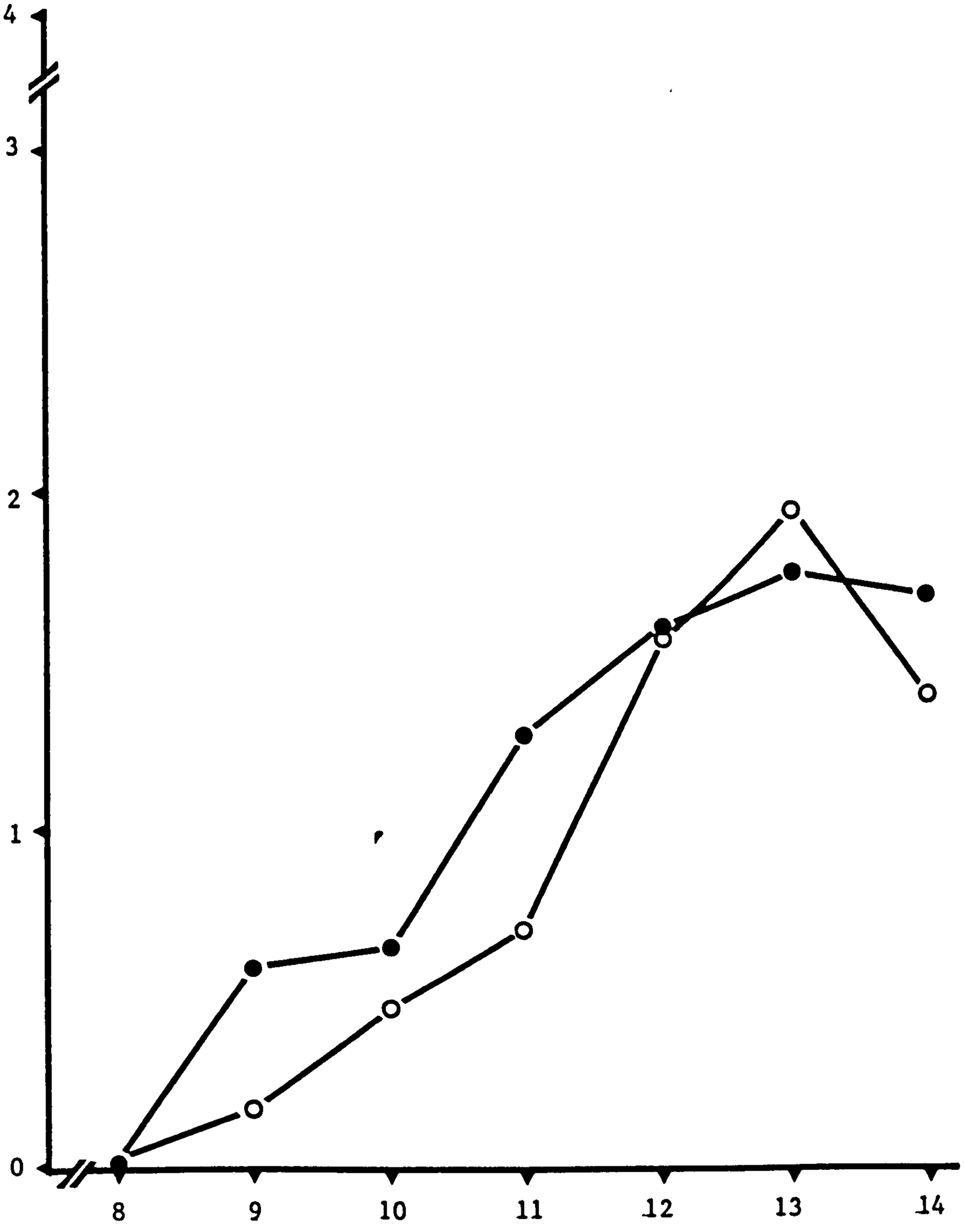


FIGURE 25. The effect of topical disodium cromoglycate on the conjunctival response to topical ovalbumin challenge between 8 and 14 days after immunization.

A batch of 120 guinea pigs was immunized with ovalbumin and divided into groups of 8 animals. Each group was topically challenged in one eye only, on one occasion between 8 and 14 days after immunization,with ovalbumin (● - ●), or ovalbumin plus disodium cromoglycate (o - o). No significant inhibition or potentiation of the conjunctival response was observed on any of these days at the dose of disodium cromoglycate tested (250 µg).

1. Topical drug vs topical histamine (visual assessment).
2. Intravenous drug vs topical histamine (visual assessment).
3. Topical drug vs intraconjunctival histamine (guinea pigs pre-treated with Evans Blue and reactions assessed by reflectometer difference value x upper conjunctival width).

The topical and intraconjunctival doses of histamine employed were chosen where possible to give conjunctival reactions of equivalent severity to those obtained with ovalbumin challenge (i.e. approximately 2+). The results for all three challenge systems are shown in Table 19.

Topical DSCG at 250 µg and 2.5 mg doses did not inhibit either topical or intraconjunctival histamine responses. In contrast, doxantrazole caused a significant reduction of responses to both topically and intraconjunctivally applied histamine at the highest topical (2.5 mg) and i.v. (100 mg/kg) doses tested. These results indicated that although DSCG and doxantrazole were found to be equally active inhibitors of the antigen conjunctival response, non-specific anti-histamine activity may possibly have contributed to the efficacy of doxantrazole at the highest topical and intravenous doses employed.

3.4. The Inhibitory Effect of a β-adrenoreceptor stimulant: Salbutamol.

The inhibitory activity of the specific β_2-adrenergic agent salbutamol against both antigen and histamine conjunctival responses was determined. The salbutamol was administered topically, incorporated in the respective challenge solutions.

Table 19. Results from three groups of experiments designed to determine the degree of non-specific inhibition of the conjunctival response to histamine exhibited by DSCG and doxantrazole.

Histamine challenge		Treatment				Reaction assessment		
Dose µg	Route	Drug	Dose	Route	n	Method	Response mean ± s.e.m.	WRST
250	Topical	Sal.	Control		10	Visual	2.1 ± 0.2	-
250	Topical	DSCG	0.25 mg	Topical	5	Visual	2.3 ± 0.3	n.s.
250	Topical	DSCG	2.5 mg	Topical	5	Visual	2.5 ± 0.4	n.s.
*75	Topical	Sal.	Control		10	Visual	1.3 ± 0.2	-
*75	Topical	Dox.	0.25 mg	Topical	5	Visual	1.2 ± 0.3	n.s.
*75	Topical	Dox.	2.5 mg	Topical	5	Visual	0.7 ± 0.4	n.s.
2	i.c.	Sal.	Control		10	WxR.D.	10.1 ± 1.2	-
2	i.c.	DSCG	2.5 mg	Topical	10	WxR.D.	7.3 ± 1.2	n.s.
2	i.c.	Dox.	0.75 mg	Topical	10	WxR.D.	8.6 ± 0.9	n.s.
2	i.c.	Dox	2.5 mg	Topical	10	WxR.D.	7.0 ± 0.7	$p<0.05$
250	Topical	Sal.	Control	i.v.	8	Visual	2.4 ± 0.3	-
250	Topical	Dox.	30 mg/kg	i.v.	8	Visual	2.6 ± 0.3	n.s.
250	Topical	Dox.	100 mg/kg	i.v.	8	Visual	1.3 ± 0.3	$p<0.05$
125	Topical	Sal.	Control		10	Visual	2.0 ± 0.2	-
125	Topical	Dox.	30 mg/kg	i.v.	10	Visual	2.0 ± 0.2	n.s.
125	Topical	Dox.	100 mg/kg	i.v.	10	Visual	1.2 ± 0.2	$p<0.02$

The effect of disodium cromoglycate (DSCG) and doxantrazole (Dox.) on the guinea pig conjunctival response to topical or intraconjunctival (i.c.) histamine was investigated. DSCG and dozantrazole were given by intravenous (i.v.) or topical route, 2 minutes prior to or at the same time as conjunctival challenge, respectively. The results are expressed as mean ± s.e.m. for visual reaction scores or width x reflectometer difference measurement (WxR.D.), and are analysed using the Wilcoxon Rank Sum Test (WRST).

* Low dose of histamine used with doxantrazole due to a 'salting out' effect at higher concentrations of histamine. n.s. : not significant Sal. : saline control.

This drug proved to be a highly effective inhibitor of the conjunctival response which follows either antigen or histamine topical challenge. A marked and dose-related inhibition of both types of challenge was observed with salbutamol doses between 0.25 and 250 µg (Figure 26). This effect was highly significant for both antigen and histamine challenge at the highest dose levels in each case ($p<0.01$).

3.5. The Activity of Prostaglandins in the Antigen Conjunctival Response.

The potential contribution of PG's to the conjunctival immediate hypersensitivity reaction was investigated by two methods. Firstly, groups of guinea pigs were pretreated with doses of the non-steroidal anti-inflammatory agent indomethacin, a recognised PG synthetase (cyclo-oxygenase) inhibitor. Secondly, PG's themselves were incorporated into intraconjunctival (i.c.) challenge solutions of ovalbumin or histamine.

(1) Indomethacin was administered either intravenously immediately prior to topical challenge (3.0 and 10.0 mg/kg), or topically simultaneously with challenge (75 µg). These doses reduced the severity of the antigen reaction in both experiments performed (Table 20), but only to a marginally significant degree ($0.1>p>0.05$) in one case.

(2) Low doses of PG's E_1, E_2, and $F_{2\alpha}$, previously established as possessing no conjunctival inflammatory effects when given alone (Chapter One of this thesis), were incorporated into histamine (1 µg) and ovalbumin (3 µg) i.c. challenge doses. PG's E_2 and $F_{2\alpha}$ (both 500 ng doses) were found to have no significant effect on either type of conjunctival challenge. Doses of PGE_1 however, in the 30 to 300 ng range, were observed to actively potentiate the conjunctival response to both ovalbumin (Figure 27) and histamine (Figure 28).

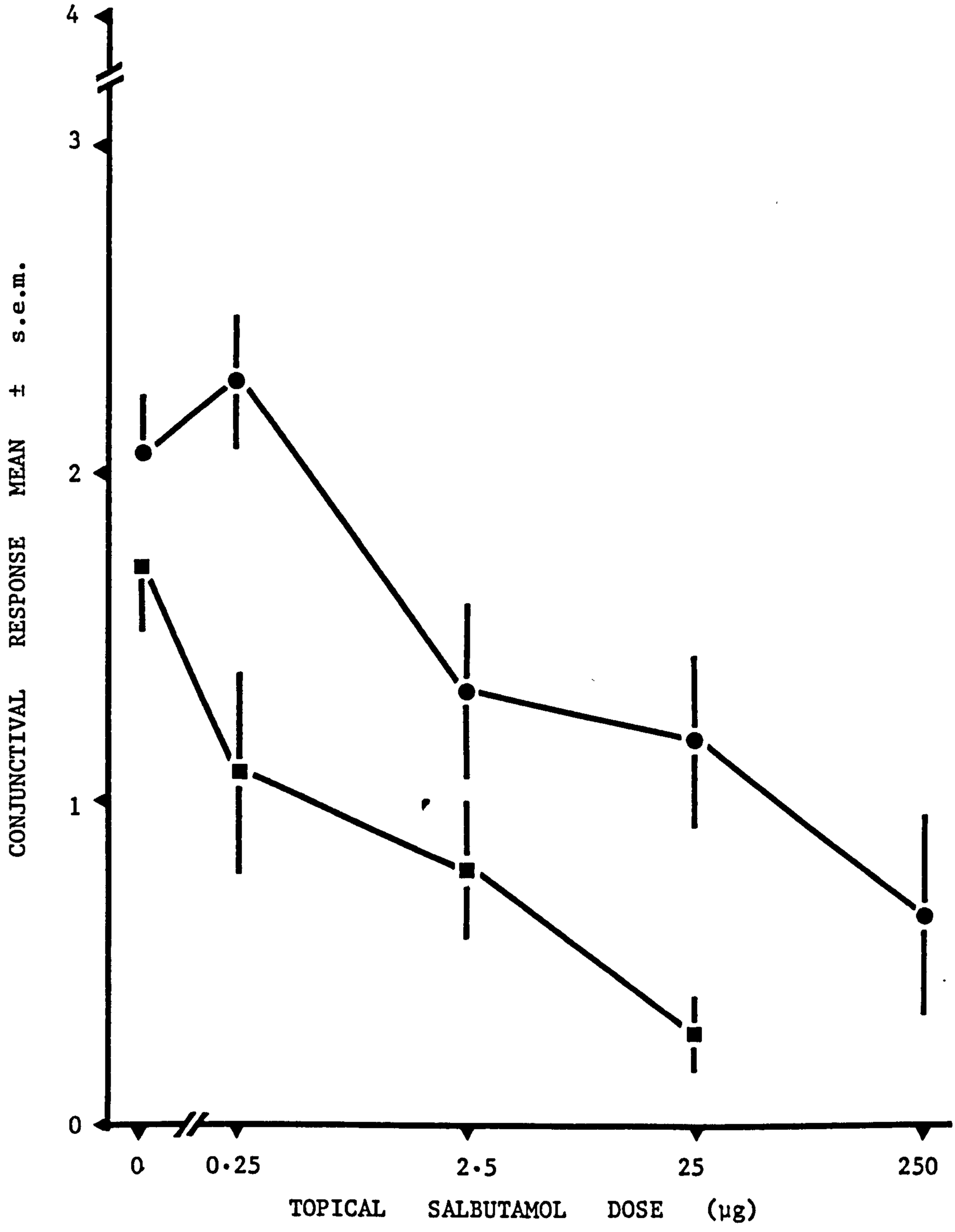


FIGURE 26. Inhibition of the conjunctival response to ovalbumin and histamine by salbutamol.

Guinea pigs were topically challenged in one eye only with ovalbumin (■ - ■) and histamine (● - ●) solutions also containing increasing doses of salbutamol (a B_2-adrenergic stimulant). Not less than 8 guinea pigs were challenged in any one treatment group.

Table 20. The effect of indomethacin on the antigen conjunctival response.

Indomethacin		C.R. (mean ± s.e.m.)		WRST
Dose	Route	Control	Test	
75 µg	Topical	1.7 ± 0.2	1.5 ± 0.3	p>0.1
3.0 mg/kg	i.v.	1.7 ± 0.2	1.2 ± 0.3	p>0.1
3.0 mg/kg	i.v.	1.6 ± 0.2	1.0 ± 0.2	p>0.1
10.0 mg/kg	i.v.	1.6 ± 0.2	0.9 ± 0.3	0.1>p>0.05

The indomethacin was administered intravenously (i.v.) via an ear vein immediately prior to challenge. All treatment groups contained not less than 10 guinea pigs. The results are expressed as mean ± s.e.m. for visually scored conjuntival responses (C.R.), and analysed using the Wilcoxon Rank Sum Test (WRST).

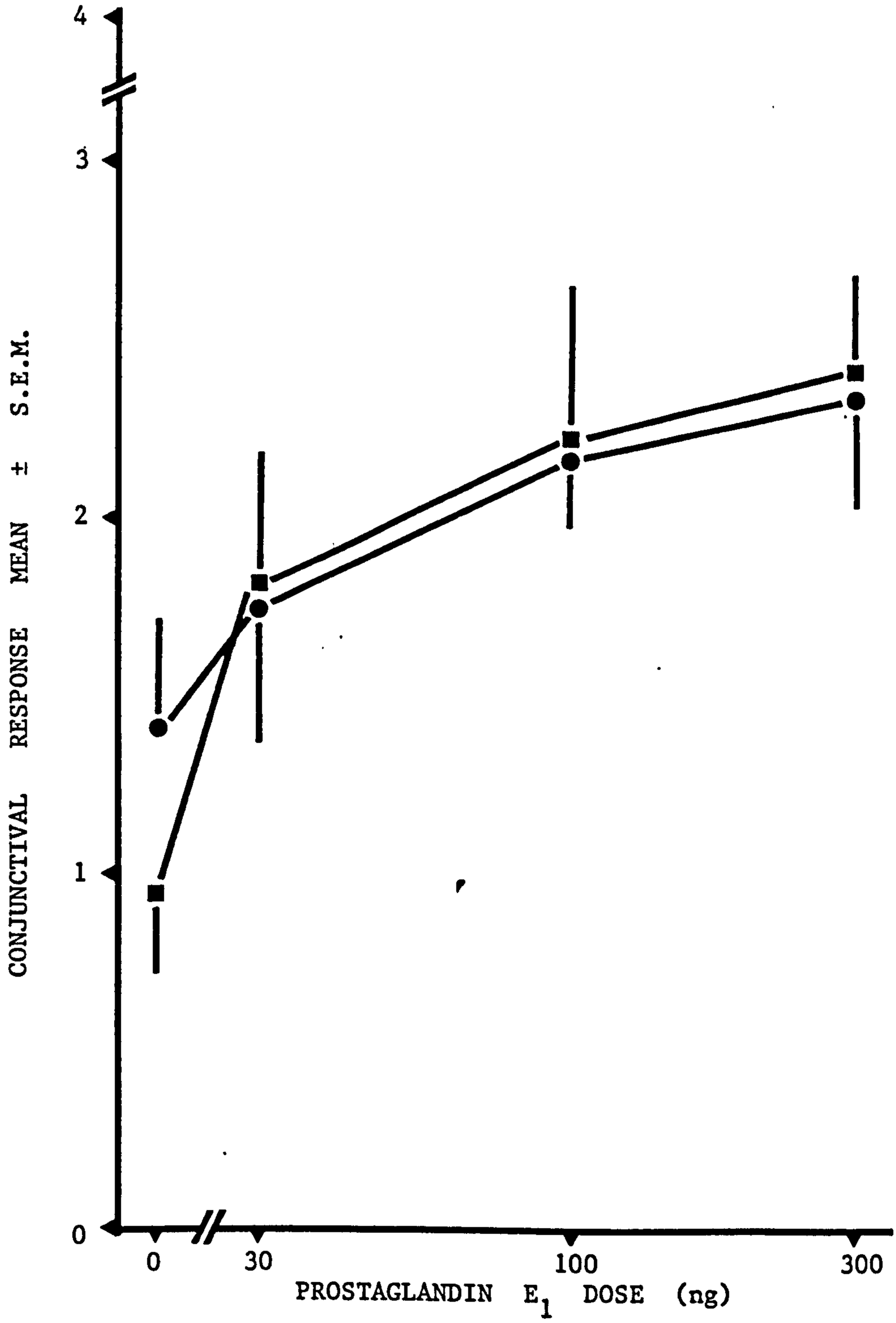


FIGURE 27. The effect of prostaglandin E_1 on the response to intraconjunctival injection of ovalbumin.

In the two experiments (● - ● and ■ - ■) shown above, increasing doses of PGE_1 were incorporated into ovalbumin solutions given by intraconjunctival injection. Five guinea pigs were challenged in each treatment group. Doses of PGE_1 between 30 and 300 ng caused no discernable conjunctival response alone (see Chapter One).

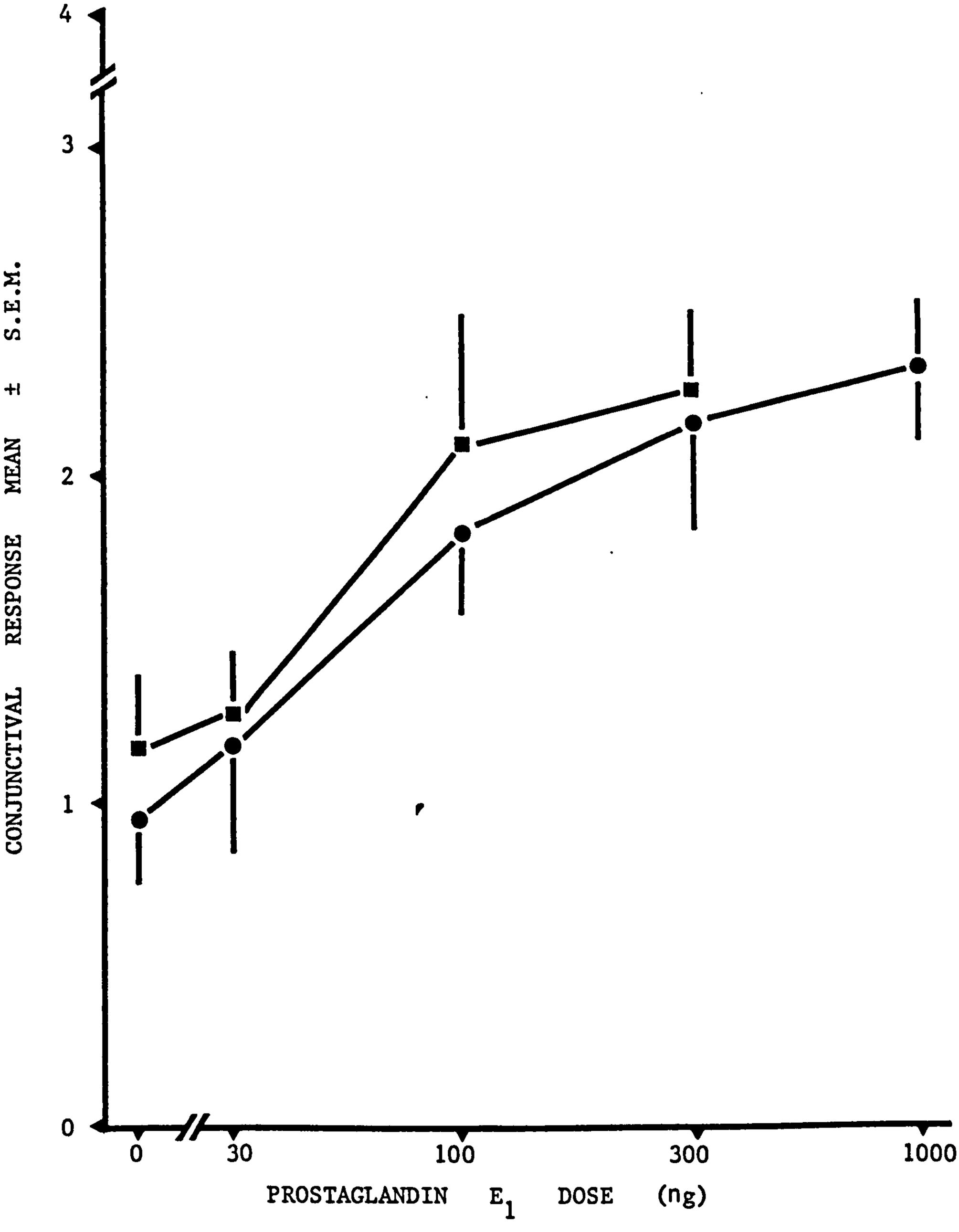


FIGURE 28. The effect of prostaglandin E_1 on the response to intraconjunctival injection of histamine.

In two experiments (● - ● and ■ - ■) increasing doses of PGE_1 were incorporated into histamine challenge solution given by intraconjunctival injection. Between 5 and 8 guinea pigs were challenged in each treatment group. Doses of PGE_1 between 30 ng and 1·0 µg caused no discernable conjunctival response alone (see Chapter One).

The effect was dose-related in both cases, and was significant at the 100 and 300 ng PGE_1 doses ($p<0.05$).

3.6. The Effect of Anaesthetics on the Conjunctival Response.

During the development of the intraconjunctival injection challenge technique, the topically applied local anaesthetic lignocaine was initially employed to alleviate any local pain or discomfort which might result. Although subsequent experiments demonstrated that pretreatment with a local anaesthetic is unnecessary in the use of this technique, a series of control experiments designed to investigate any effect of lignocaine on the conjunctival response itself produced some interesting results.

A topical 5 minute pretreatment with lignocaine (1.0 mg) was found to cause a significant reduction in the antigen conjunctival response (Table 21). In contrast, no effect was observed on the histamine response, whereas significant potentiation of the compound 48/80 response was obtained on two occasions (Table 21).

In a single experiment, it was confirmed that systemic anaesthesia induced with pentabarbitone (60 mg/kg) has no effect on the conjunctival response to topically instilled antigen.

3.7. The effect of Dexamethasone on the Conjunctival Response

An experiment was performed to investigate the inhibitory effect of a representative anti-inflammatory steroid, dexamethasone, on the antigen an histamine conjunctival responses. Groups of guinea pigs were pre-treated with dexamethasone (10.0 mg/kg) or saline by intraperitoneal

Table 21. The effect of local and systemic anaesthesia on the conjunctival anaphylactic response.

	Challenge System			Conjunctival response mean ± s.e.m.		
Anaesthetic	Challenge	Dose	n	Control	Test	W.R.S.T.
LIGNOCAINE	OVALBUMIN	500 µg	16	2.3 ± 0.1	1.3 ± 0.2	p<0.01
LIGNOCAINE	OVALBUMIN	500 µg	11	2.2 ± 0.2	1.5 ± 0.4	p<0.05
LIGNOCAINE	HISTAMINE	375 µg	10	2.7 ± 0.1	2.6 ± 0.1	n.s.
LIGNOCAINE	HISTAMINE	250 µg	10	2.1 ± 0.2	2.1 ± 0.2	n.s.
LIGNOCAINE	COMPOUND 48/80	7.5 mg	15	*0.4 ± 0.1	2.4 ± 0.2	p<0.001*
LIGNOCAINE	COMPOUND 48/80	7.5 mg	10	1.1 ± 0.2	2.5 ± 0.2	p<0.001
PENTABARBITONE	OVALBUMIN	500 µg	10	2.4 ± 0.2	2.7 ± 0.3	n.s.

LIGNOCAINE (1.0 mg) was applied topically to the conjunctiva 5 minutes before challenge.

PENTABARBITONE (nembutal): 60 mg/kg was given by intraperitoneal injection 30 minutes before challenge.

Results were expressed as mean ± s.e.m. for visually scored conjunctival responses, and analysed using the Wilcoxon Rank Sum Test (W.R.S.T.).

* A low control compound 48/80 response was obtained in this experiment, thus possibly over emphasing the potentiating effect of compound 48/80, on a significantly sub-maximal control response.

injection at 4 or 24 hours prior to topical challenge with either histamine or ovalbumin. No significant inhibition of either type of conjunctival reaction was observed in those guinea pigs given dexamethasone 24 hours before challenge (Table 22). At 4 hours however, dexamethasone at this dose was effective aginst the histamine but not the ovalbumin response (Table 22).

Table 22. The effect of dexamethasone on the conjunctival response.

Challenge	Pretreatment (hours)	C.R. (mean ± s.e.m.) Control	Test	WRST
Ovalbumin	4	1.8 ± 0.2	1.7 ± 0.3	n.s.
Ovalbumin	24	2.1 ± 0.3	1.9 ± 0.3	n.s.
Histamine	4	2.5 ± 0.3	1.1 ± 0.2	$p<0.01$
Histamine	24	2.6 ± 0.3	2.3 ± 0.3	n.s.

Guinea pigs were pretreated with dexamethasone (10 mg/kg) in celacol, or saline (controls), given by intraperitoneal injection at 4 or 24 hours before topical challenge. Conjunctival responses (C.R.) were scored visually and analysed using the Wilcoxon Rank Sum Test (WRST). Each treatment group contained not less than 10 guinea pigs.

n.s. : not significant

D I S C U S S I O N

Triprolidine proved to be a potent inhibitor of antigen, histamine, and compound 48/80 induced conjunctival reactions when given either topically (25 µg), or intraperitoneally (1-3 mg/kg). These results are broadly in agreement with previous reports that systemic triprolidine doses between 0.1 and 10 mg/kg protect guinea pigs from antigen induced anaphylactic shock (Armitage, Herxheimer, and Rosa, 1952; Green, 1953), and inhibit both IgG_1 and IgE mediated guinea pig PCA reactions (Movat et al., 1967; Follenfant, unpub. obs.). In addition, Dwyer, Darougar, and Jones (1976) have reported complete inhibition of guinea pig conjunctival anaphylaxis with systemic (2.0 mg/kg) and topical (one drop of a 10 mg/ml solution) doses of triprolidine.

The total inhibition of the histamine conjunctival response by triprolidine (Figures 20 and 21) strongly indicated that this response is mediated exclusively via H_1 histamine receptors. As only partial inhibition of the antigen conjunctival response was recorded in the present study (Figure 20), even at the highest triprolidine doses tested, the results implied that additional anaphylactic mediators apart from histamine are released. These may include SRS-A (Brocklehurst, 1960; Stechschulte, Orange, and Austen, 1973), eosinophil chemotactic factor of anaphylaxis (Kay and Austen, 1971), E and F series PG's (Horton, 1969; Piper and Vane, 1969), and thromboxane A_2 (Vane, 1976).

Experiments designed to test (1) the conjunctival response to topical challenge with purified SRS-A preparations, and (2) the inhibitory activity of the recently reported specific SRS-A receptor antagonist FPL 55712 (Augstein et al., 1973), would provide important information concerning a possible role for SRS-A in guinea pig conjunctival anaphylaxis.

Partial inhibition of the conjunctival response to ovalbumin was observed with the 5-HT receptor competitive antagonists B.W. 501C67 and methysergide. However, since significant inhibition of the histamine response also occurred at the effective doses of these drugs (Table 18), the specificity of receptor antagonism at the doses employed remains open to question.

Significant potentiation of the conjunctival response to both antigen and histamine was obtained when low PGE_1 doses (30-300 ng) were incorporated into intraconjunctivel challenge solutions. PGE's have been previously observed to potentiate histamine and bradykinin induced increases in guinea pig cutaneous permeability (Williams and Morley, 1973), and carrageenin induced paw oedema in the rat (Moncada, Ferreira, and Vane, 1973). Vane (1976) proposed that rather than contributing directly to local erythema or oedema formation, PG's may act by increasing the inflammatory effects of other known mediators.

Conjunctival PG synthetase activity in the rabbit has been found to be high compared to that of retina, anterior uvea, spleen, and kidney medulla in the same species (Bhattacherjee and Eakins, 1974). The authors concluded that in the rabbit, PG's may be active in external conjunctival inflammation, in addition to uveitis as previously reported (Eakins et al., 1972).

In the guinea pig conjunctiva, although the response potentiating effects of PGE_1 were clearly apparent, PG's alone produced no response (Chapter One of this thesis). The inhibitory effects of systemic indomethacin pretreatment were also only marginal (Table 20). No conclusive evidence was therefore obtained for a significant PG contribution to conjunctival anaphylaxis in the guinea pig.

The contradictory results obtained during DSCG inhibition studies reported in the literature have emphasised that at least three important criteria must be considered when comparing the activity of this drug in various test systems. Firstly, the target tissue chosen is important, e.g. skin, lung, or leucocytes. Secondly, the experimental method employed may affect the results, i.e. actively vs. passively sensitized tissues, challenged *in vivo* or *in vitro*. Thirdly, the nature of the homocytotropic anaphylactic antibody responsible appears critical, as reagin (IgE) mediated systems appear more sensitive to DSCG than those mediated by IgG_1 (guinea pig) or IgGa (rat).

Previously reported results in the guinea pig have emphasised the importance of the above mentioned considerations. For example, DSCG is inactive against IgG_1 mediated guinea pig PCA (Cox, 1967; Lopez and Bloch, 1969), and histamine release from passively sensitized skin slices (Yeoh, Tay, and Greaves, 1972). In contrast, IgE mediated PCA in the guinea pig is highly sensitive to DSCG (Taylor, 1973; Taylor and Roitt, 1973). In lung, Cox (1967) observed no inhibition of histamine from actively or passively sensitized tissue by DSCG, whereas Assem and Richter (1971) found this drug to be active against histamine release mediated by a purified antidinitrophenol IgG_1 antibody. Furthermore, DSCG has also been shown to inhibit histamine release from blood basophils in this species (Greaves, 1969).

Resolution of the results described in my own and previous studies has been further complicated by the recognition of two distinct guinea pig IgG_1 homocytotropic antibody populations, in addition to IgE (Parish, 1970c; Ovary and Warner, 1972; Perini and Mota, 1972). Anaphylactic reactions mediated by each of these homocytotropic antibodies could

conceivably possess widely differing sensitivities to DSCG. Furthermore, a combination of all three may contribute to immediate hypersensitivity reactions in the guinea pig conjunctiva (see Chapter Two).

In the experiments described here (Figure 23), both DSCG and doxantrazole significantly inhibited the ovalbumin conjunctival response when applied topically (750 µg and 2.5 mg doses). On the other hand, doxantrazole was also inhibitory when given intravenously (30 and 100 mg/kg), whereas DSCG was not. However, doxantrazole was also shown to possess non-specific anti-histamine activity at the highest doses tested, which presumably contributed to its slightly greater potency in each test.

Taylor and Roitt (1973) have previously reported DSCG inhibition of guinea pig conjunctival anaphylaxis at 9, 10, and 11 days after ovalbumin immunization, and slight potentiation on days 13 and 14. Using my own immunization and challenge protocols, I have been unable to confirm either the inhibition or potentiation observed by the above authors. This may be due to a number of important differences in the experimental designs, which include dose and route of antigen immunization, and the topical antigen and drug doses employed.

In the present study, the β_2-adrenoreceptor stimulant salbutamol proved to be a highly effective inhibitor of both antigen and histamine induced conjunctival reactions. The activity of salbutamol against the antigen response can at least in part be explained by the recognised inhibitory effects of β-adrenergic agents on mast cell mediator release. However, since highly significant inhibition of the histamine conjunctival response was also observed, this cannot be the complete explanation. Two recent reports describing the effects of β-adrenergic agents on skin wheal and flare (Shereff et al., 1975) and skin histamine (Jorde and

Schata, 1976) responses in man have both concluded that these drugs may also affect small blood vessels directly, probably via cAMP. A direct effect on the local conjunctival vasculature might therefore be the mechanism by which salbutamol antagonised the histamine response in my own work. Some additional experiments incorporating β-receptor blockade (e.g. propranolol) would at least elucidate whether this is a specific β-receptor effect.

The series of experiments designed to investigate the effects of the local anaesthetic lignocaine on the conjunctival response were initially undertaken on the basis that challenge by intraconjunctival injection might require induction of temporary local anaesthesia. In the event, guinea pigs were found to tolerate this technique satisfactorily, without requiring pretreatment with lignocaine. Nevertheless, the contrasting effects of lignocaine on the conjunctival response, causing inhibition of the antigen reaction and potentiation of the compound 48/80 response, aroused considerable interest.

Local anaesthetics of this type are usually esters (e.g. cocaine or procaine) and amides (e.g. lignocaine) based on p-amino benzoic acid. Conventional drug-receptor theories do not explain their action, which involves preventing the generation and conductance of nerve impulses. This is believed to occur through an as yet undefined adverse effect on the nerve membrane lipid bilayer, which has the result of altering or closing the membrane pores through which ions move in the propagation of action potentials (Ritchie and Cohen, 1978).

The concept that conformational changes in the structure of the cell membrane may be induced by local anaesthetics has been widely recognised (Seeman and Roth, 1972; Rabinovitch and De Stefano, 1976). These agents

have been shown to 'fluidize' erythrocyte cell membranes (Goldstein, Aronow, and Kalman, 1974), and to cause gel to liquid crystalline phase transitions in artificial dipalmitoyl lecithin liposomes (Jain, Wu, and Wray, 1975).

A local anaesthetic such as lignocaine might therefore be expected to have important effects on a variety of cell membrane-dependent physiological functions. This does appear to be the case. For example, it has been shown to antagonise histamine induced contraction of guinea pig tracheal smooth muscle *in vitro* (Weiss, Anderson, and O'Brien, 1975). This action, unaffected by either β-adrenergic (propranolol) or cholinergic (atropine) blockade, was attributed by the above authors to a stabilizing effect on smooth muscle membrane via a reversible interference with calcium ion flux. Lignocaine has also been observed to significantly reduce both random and chemotactic factor stimulated locomotion in blood leucocytes (Moudgil et al., 1977) and activation of lymphocytes *in vitro* (Waterfield, Hanmarstrom, and Smith, 1976). Of greater relevance to the present study is its reported ability to inhibit compoud 48/80 induced histamine release from rat peritoneal mast cells (Kazimierczak, Peret, and Maslinski, 1976), although in the same type of experiment, the closely related substance tetracaine possessed inhibitory activity at low doses while enhancing release at higher concentrations.

In the guinea pig conjunctiva, the contrasting effects of lignocaine on the antigen and compound 48/80 but not histamine responses strongly suggested that its site of action is at the mast cell. No definite conclusions may be drawn from the data obtained in the present study as to the mechanism of action of lignocaine. In the context of mediator release from mast cells however, adverse effects on membrane

integrity might be expected to affect either the initial cell membrane activation step which follows antigen-antibody combination, or the subsequent intracellular rearrangement of microtubules and granular membranes. The lack of any discernable effect of systemic pentabarbitone anaesthesia on the conjunctival antigen response is in agreement with the previous observation of Parish, Hall, and Coombs (1963), who found that whole animal anaesthesia induced with either volatile inhalation anaesthetics or pentabarbitone has no effect on systemic guinea pig anaphylaxis.

The beneficial effects of adreno-corticosteroid therapy in allergic conditions are well established. For example, hydrocortisone, prednisone, and dexamethasone, have been widely used to provide relief from the symptoms of asthma and hay fever (Baldwin, Dworetzky, and Isaacs, 1961; Cameron et al., 1973). A new topically applied steroid, beclomethasone, has also recently proved effective, without the commonly occurring side effect of adrenal suppression (Mygind, 1973; Gibson et al., 1974; Morrison-Smith et al., 1975).

A short term pretreatment of guinea pigs with dexamethasone at 4 and 24 hours before challenge had no effect on the antigen conjunctival response in the present study. A small but significant effect on the histamine response was observed with a 4 hour dexamethasone pretreatment, for which there appears no obvious explanation in view of its inactivity against the antigen reaction. Although single doses of dexamethasone have been reported as being effective in the guinea pig (Kurihara and Shibata, 1975), it is widely thought that treatment with cortisone or dexamethasone must be continued over an extended period of 14-28 days before significant beneficial effects (such as a fall in tissue histamine levels) will occur (Telford and West, 1960; Hicks, 1965; Kovacs, 1965).

Dwyer, Darougar, and Jones (1976) have also previously found the guinea pig conjunctival anaphylactic response insensitive to systemic doses of cortisone (25 mg/kg) given at 24 hours and 20 minutes before challenge.

CHAPTER FOUR

HISTOLOGICAL STUDIES ON THE GUINEA PIG CONJUNCTIVA

I N T R O D U C T I O N

The presence in the bloodstream or at specific tissue sites of abnormally high numbers of eosinophils, neutrophils, or lymphocytes, has long been recognised as characteristic of a wide range of human clinical disorders, including immunological hypersensitivity reactions.

Delayed (Type IV) hypersensitivity reactions in skin are recognisable histologically by a transient appearance of neutrophils (Long, Vorwald, and Donaldson, 1931; Angevine and Seastone, 1950), and a subsequent dense mononuclear infiltration (Dienes and Mallory, 1932; Gell and Hinde, 1951; Uhr, 1966; Turk, 1975). A neutrophil infiltration is also closely associated with the formation of immune-complexes, and complement activation typical of Arthus (Type III) lesions (Ovary, 1958; Cochrane, Weigle, and Dixon, 1959; Cochrane and Aiken, 1966).

Systemic or local eosinophilia is frequently observed in a number of clinical conditions including the allergic diseases, skin disorders, parasitic infections, and eosinophilic leukaemia. Eosinophils are commonly present in bronchial sputum smears from asthmatics, and in nasal and conjunctival secretions of patients suffering from allergic rhinitis and conjunctivitis (Loveless, 1945; Stromme, 1955; Fontana, Spain, and Desanctis, 1956; Morrow-Brown, 1958; Connell, 1968; Parish and Pepys, 1968; Smith, Casanova-Roig, and Wells, 1968; Ishizaka and Newcomb, 1970; Fujita et al., 1975).

Although skin testing has been extensively used in clinical practice to identify specific allergens, little was known about the histological changes which occur during and following a typical skin wheal and flare reaction until comparatively recently. Kline, Cohen,

and Rudolph (1932) were the first to observe an eosinophilic and neutrophilic infiltration into skin sites of atopic subjects tested with ragweed allergen. Development of the 'skin window' technique for the study of local cellular infiltration (Rebuck and Crowley, 1955; Hu, Fosnaugh, Bryan, and Jacks, 1961) has confirmed an active infiltration of eosinophils and neutrophils into human skin test sites over the 24 hour period following allergen challenge. The neutrophil response appears faster in onset, with large numbers of these cells being present as early as 30-60 minutes after challenge. Eosinophil accumulation commences more slowly, numbers being low at 4 hours, and increasing between 12 and 24 hours (Eidinger, Raff, and Rose, 1962; Eidinger, Wilkinson, and Rose, 1964; Fowler and Lowell, 1966; Feinberg, Feinberg, and Lee, 1967; Atkins, Green, and Zweiman, 1973).

Local tissue eosinophilia in man and experimental animals is therefore widely recognised as frequently being a direct result of anaphylactic reactions. Consequently, eosinophil infiltrations are often most dense in those tissues which contain numerous mast cells, and where anaphylactic mediators have been released following antigen or compound 48/80 application.

In the guinea pig, eosinophils have been found in large numbers in the peritoneal exudate after repeated intraperitoneal injections of foreign proteins (Litt, 1960a, 1960b), or antigen-antibody complexes (Litt, 1961, 1962), and as a result of passive peritoneal anaphylaxis (Redd and Vaughan, 1955). Eosinophils may also be indirectly involved in primary antigen recognition, as they have been observed to accumulate in the axillary and popliteal lymph nodes of guinea pigs within 4 hours of antigen injection into the footpads (Litt, 1964b).

As in man, both eosinophils and neutrophils infiltrate skin sites in guinea pigs during the 24 hour period after active or passive cutaneous anaphylaxis, whether IgE or IgG_1 antibodies are responsible (Kay, 1970a; Parish, 1970a, 1972a, 1972b; Muller and Healy, 1973).

Cellular infiltration has also been previously demonstrated in the guinea pig conjunctiva following topical serum antigen challenge. Although originally observed to comprise a predominantly eosinophilic response from 24-48 hours after challenge (Feinberg, unpub. obs.), more recent reports have described a mixed cellular infiltration of both eosinophils and neutrophils commencing within 60 minutes of rabbit serum challenge, and lasting for 48-72 hours (Dwyer and Darougar, 1971; Dwyer, Turk, and Darougar, 1974).

The purpose of the present work was therefore to re-investigate the nature of the cellular infiltration which follows guinea pig conjunctival anaphylaxis, in terms of both distribution and quantification of eosinophil and neutrophil response, and to compare antigen-induced cellular infiltration with that which follows histamine or compound 48/80 challenge.

MATERIALS AND METHODS

Conjunctival smears:

Smears were taken by loop from the conjunctivae of guinea pigs at 24 hours after topical challenge. The smears were air dried or alcohol fixed, before staining with Hansel stain (Lide Laboratories Ltd., St. Louis, Missouri, U.S.A.). This commercial diagnostic preparation was found to be a highly effective stain for both eosinophils and neutrophils. The smears were then washed in 70% alcohol and permanently mounted in Duramount (G.T. Gurr Ltd.).

Each smear was assessed for cellular content by differential counts of those cells observed in random sweep fields at x 400 magnification. A minimum of 200-300 cells were counted per smear. The results are expressed for each cell type as a percentage of the total number of cells counted on a given smear. Appropriate controls (smears from unchallenged or saline challenged eyes) were incorporated in all experiments.

Paraffin sections:

Paraffin sections (7μ) were employed to investigate the general structure of the guinea pig conjunctiva, the distribution of mast cells, and to define and quantify more fully the eosinophilic and neutrophilic infiltration initially indicated by conjunctival smears.

Whole eyes were removed at sacrifice either from normal guinea pigs (for structural and mast cell studies), or at various intervals after topical conjunctival challenge. The control treatments used in the cellular infiltration investigation were of three types:

(1) 'normal' eyes removed from guinea pigs unused in any previous

experimental procedure.

(2): saline challenged guinea pig conjunctivae.

(3): 'normal' guinea pigs topically challenged with antigen.

The whole eyes were fixed according to the cell types to be studied. For the general structure and cellular infiltration work, formol-alcohol was routinely used (50 ml 10% formalin, 450 ml 90% alcohol, and 25 ml glacial acetic acid). Three types of fixative were tested in order to find a successful formula for mast cell fixation in guinea pig tissues:

(i) A mercuric chloride sublimate fixative (5.5g mercuric chloride and 2.7g sodium acetate dissolved in 100 ml distilled water) originally described by Donaldson et al., (1973).

(ii) 4% w/v basic lead acetate in 50% ethanol (Parish, 1964).

(iii) Formol-alcohol fixative as above.

After 24-48 hours in fixative, the eyes were transferred to, and stored in 70% alcohol until processing. The optical stalk, retina, sclera, vitreous body, and lens, were then cut away, and the remaining tissue (eyelids, conjunctiva, cornea, and iris) processed and blocked up in paraffin wax for sectioning.

Those sections used for general structure and cellular infiltration studies were stained by conventional methods using haematoxylin and eosin. Sections fixed for mast cell distribution work were stained with 1% Toluidine Blue (G.T. Gurr Ltd.) using a modification of the technique of Parish (1964). Briefly, the sections were deparaffinised and brought to 70% alcohol in the usual way, before being stained with 1% Toluidine Blue in 70% alcohol for one hour. The sections were then blotted to

remove excess stain, washed in 70% alcohol, dehydrated in absolute butanol rather than absolute ethanol (2 x 1 minute), and cleared in xylol before mounting.

Assessment of Cellular Infiltration:

The eosinophilic and neutrophilic infiltration into the conjunctival tissue was assessed in two ways:

(1) Tissue mapping of the cellular distribution on each individual section, and subsequent estimation of eosinophil and neutrophil numbers present using a 0 to 3+ scoring system defined as follows:

SCORE

0 No polymorphonuclear cells present.

1 Scattered cells present in the sub-mucosal conjunctival tissue.

2 Eosinophils or neutrophils regularly distributed in the sub-mucosal tissue and mucosal epithelium.

3 Large numbers of cells observed throughout the sub-mucosal tissue and along the mucosal cell layer, particularly in the basement membrane region.

(2) Localized eosinophil and neutrophil counting along the length of the conjunctival mucosal epithelium, and in the immediate sub-mucosal tissue within 0.3 mm of the mucosal basement membrane (defined by graticule).

Sections prepared from guinea pig eyes were cut and stained in batches, up to fifty being processed at any one time. They were routinely coded at the fixation stage to ensure that assessment was performed 'blind', whichever of the above two methods was used.

The methods of guinea pig immunization, challenge, and reaction assessment used were as described in Chapter One. Standard topical doses for conjunctival challenge were as follows:

1. antigen (ovalbumin): 500 µg.
2. histamine : 250 µg.
3. compound 48/80 : 7.5 mg.

R E S U L T S

4.1. Histology of the Guinea Pig Eye.

Following the removal of the retina, sclera, lens, and vitreous body of the eye at the tissue fixation stage, each stained and mounted section consisted of the upper and lower eyelids and conjunctivae, the cornea, and the iris (including the ciliary body). A schematic half section diagram of one eyelid, conjunctiva, and cornea is shown in Figure 29.

The conjunctiva itself consists of two main tissues, the palpebral conjunctiva which lines the inner surface of the eyelids, and the bulbar conjunctiva which covers the 'white' of the eyeball. The conjunctival mucosal cell layer is of typical stratified columnar epithelial cells, with superficial flat cuboidal type cells and deeper columnar cells making up a mucosal epithelial layer 3 to 4 cells thick in all. At each end of the conjunctival mucosa is an admarginal zone, where the stratified columnar epithelial cells of the conjunctival mucosa change either into the cornified epidermal cells of the outer eyelid, or into the squamous epithelial cells of the cornea. The reflection of the palpebral and bulbar conjunctival tissues forms two deep recesses between the upper and lower eyelids and the eyeball. These two regions are known as the superior and inferior fornices, where the columnar epithelium is occasionally observed to thicken slightly. Beneath the outer mucosal epithelial cell layer lies a basement membrane, which divides the mucosa from the submucosal connective tissue (termed the palpebral fascia).

The funcion of the conjunctiva is to clean, moisten, and protect the surface of the eye, using tear fluid secreted from the lachrymal

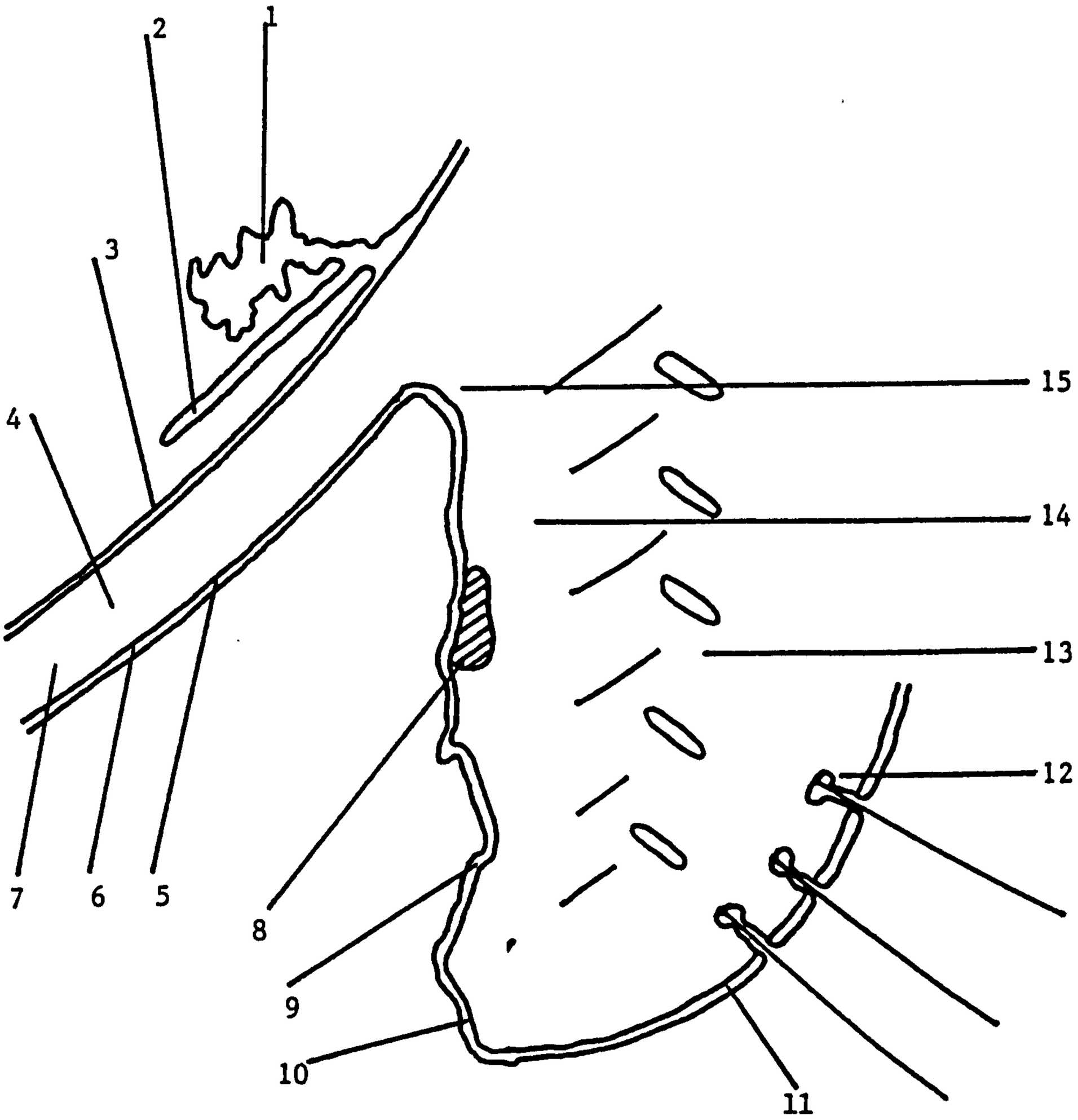


FIGURE 29. A half section diagram of the eyelid, conjunctiva and cornea of the guinea pig.

Key:
1. Ciliary process and muscle
2. Iris
3. Descamet's membrane and posterior corneal epithelium
4. Substantia propria
5. Bowman's membrane
6. Squamous epithelial cell layer
7. Cornea
8. Lymph node
9. Palpebral conjunctiva (stratified columnar epithelium)
10. Mucosal basement membrane
11. Epidermis
12. Hair follicle
13. Orbicularis muscle
14. Sub-mucosal tissue (palpebral fascia).
15. Region of the fornix

glands, and mucous from the secretory cells in the conjunctival epithelium. Being an externally exposed mucous membrane, the conjunctiva is required to serve as a first line of defence against a whole variety of environmental hazards, including airborne pollens and other common allergens.

The submucosal connective tissue is provided with a dense capillary network and lymphatic system, which in the guinea pig, includes a lymph node in the fornix region of one or both eyelids. It is this abundant vascular and lymphatic system which accounts for both the rapid absorption of antigens or drugs placed in the conjunctival sac, and the intense oedema and erythema characteristic of guinea pig conjunctival anaphylaxis and human hay fever.

The cornea is a transparent non-vascular membrane consisting of five main types of cell layer. The outer cell layer is composed of squamous epithelial cells, 4-5 cells thick, and is a very sensitive tissue. It is known to possess free nerve endings of the pain type, which are important in the blinking reflex, and in the production of tear fluid. The corneal outer squamous epithelium rests on a layer of cells known as the Bowman's membrane. The bulk of the cornea is made up of the substantia propria, a dense connective tissue which contains both cells and extracellular substance. The flattened connective tissue cells lie between bundles of collagenic fibres. Wandering lymphoid cells are sometimes present, especially during inflammation, and are thought to arise from the blood vessels of the corneal limbus. The two innermost layers of the cornea are the basement membrane (referred to as the Membrane of Descamet), and a layer of mesenchymal epithelium lying over the basement membrane termed Descamet's epithelium.

The iris is a loose, pigmented, and highly vascular tissue, the smooth muscle of which controls the diameter of the pupil, and hence the amount of light entering the eye. The iris is connected to the ciliary body, itself consisting of the ciliary muscle and process, by a tissue known as the ciliary margin. The function of the ciliary body is to help control the focal length of the lens.

The outermost epidermal layer of the eyelid or skin is provided with many small hairs and sebaceous glands. The follicles of these hairs penetrate deeply into the dermis, which contains blood vessels, nerves, and fibroblasts, but only rarely reticulo-endothelial cells. The orbicularis muscle, responsible for the closing of the eyelids, is comprised of pale striated fibres situated between the inner conjunctival submucosal connective tissue and the outer subcutaneous layers of the eyelid.

4.2. The distribution of Mast Cells in the Guinea Pig Conjunctiva.

The method of tissue fixation employed is critical for the study of mast cells in many species, and not least in the guinea pig, where the mast cell granules are known to possess water soluble tendencies. This problem is normally overcome by the use of either high alcohol content or lead and mercury salt fixatives. However, the latter type of salt fixative did not prove very successful in the present investigation. The lead fixative in particular was found to leave a crystalline deposit in the tissue which was difficult to remove. The same formol-alcohol fixative as employed in the structural and cellular infiltration studies eventually gave the most satisfactory results when combined with the modified Toluidine Blue staining technique.

The mast cells identified in the guinea pig conjunctiva (Figure

30) were compared with those characteristically found in guinea pig subcutaneous connective tissue preparations (Figure 31). The mast cells present in both tissues stained metachromatically dark purple due to the heparin laden granules present in this cell type. The background tissue stained a non-specific pale blue in each case.

The mast cells observed in subcutaneous connective tissue and conjunctival submucosa were similar in appearance, often being irregular in shape, and possessing discernable purple granules under the light microscope. In the guinea pig conjunctiva, the mast cells were distributed regularly, but not in large numbers (perhaps ten to twenty cells per section), along the length of the palpebral submucosal tissue. Mast cells were not observed particularly lying adjacent to blood vessels as occurs in some tissues, and none were found in the deeper submucosa or in the subcutaneous tissue of the eyelid.

4.3. Cellular Infiltration into the Conjunctiva Following Topical Challenge.

4.3.1. Conjunctival smears:

The smears were taken from the conjunctivae of guinea pigs at 24 hours after topical challenge with antigen, histamine, or compound 48/80. Preliminary experiments confirmed the presence of neutrophils and eosinophils in smear material, but the results were inconclusive due to a combination of lack of material on some smears, and poor staining quality on others. Improvement of the smearing technique with practice, together with the introduction of Hansel stain (found to be particularly effective for eosinophils), resulted in cell counting data typical of that shown in Table 23.

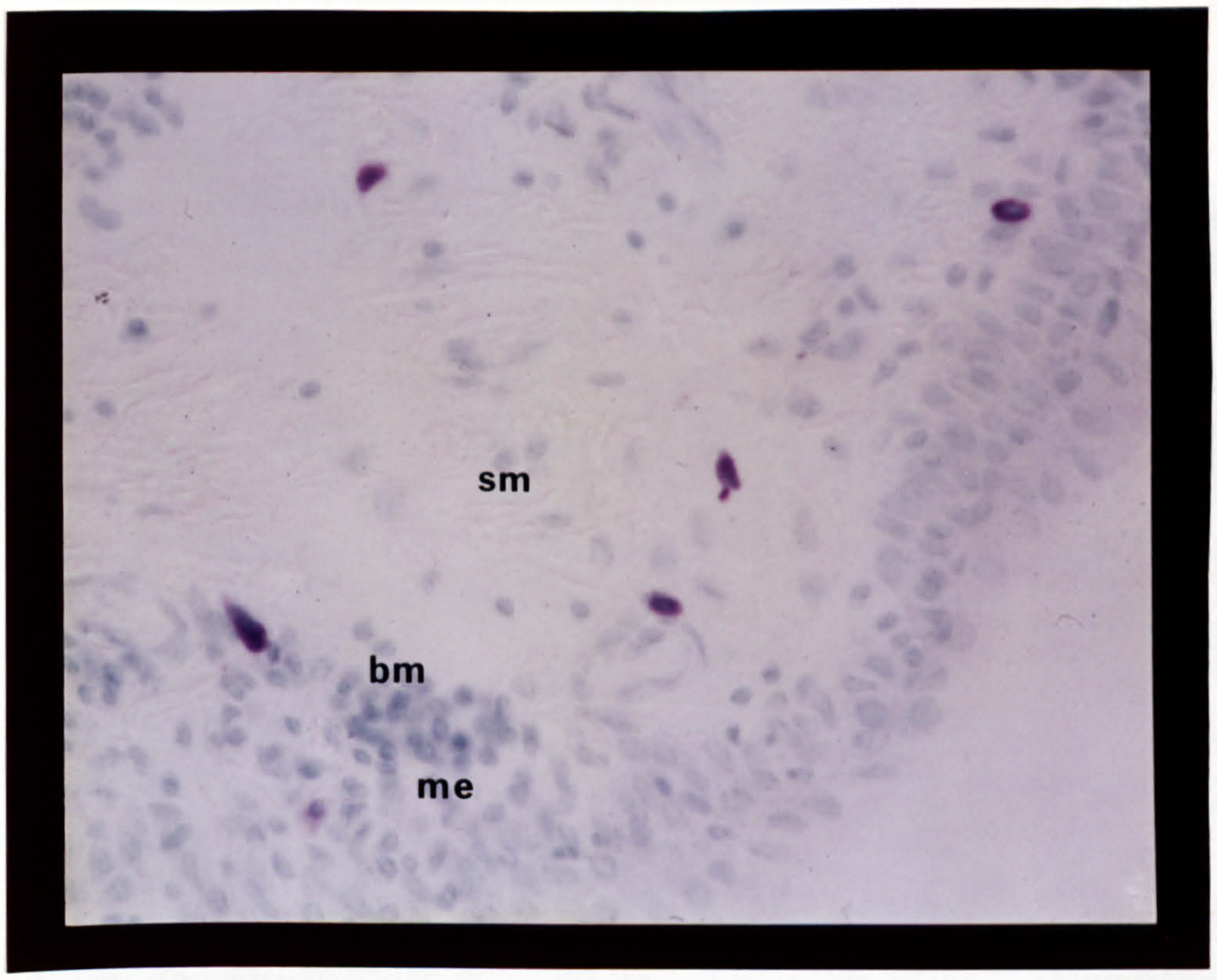


Figure 30. Mast cells in the guinea pig conjunctiva.

Mast cells were present in the submucosal connective tissue (sm) of the guinea pig palpebral conjunctiva, often close to the mucosal epithelial basement membrane (bm). None were observed in the mucosal epithelium (me) itself. The section was fixed in formol-alcohol, and stained with 1% Toluidine Blue (x 400).

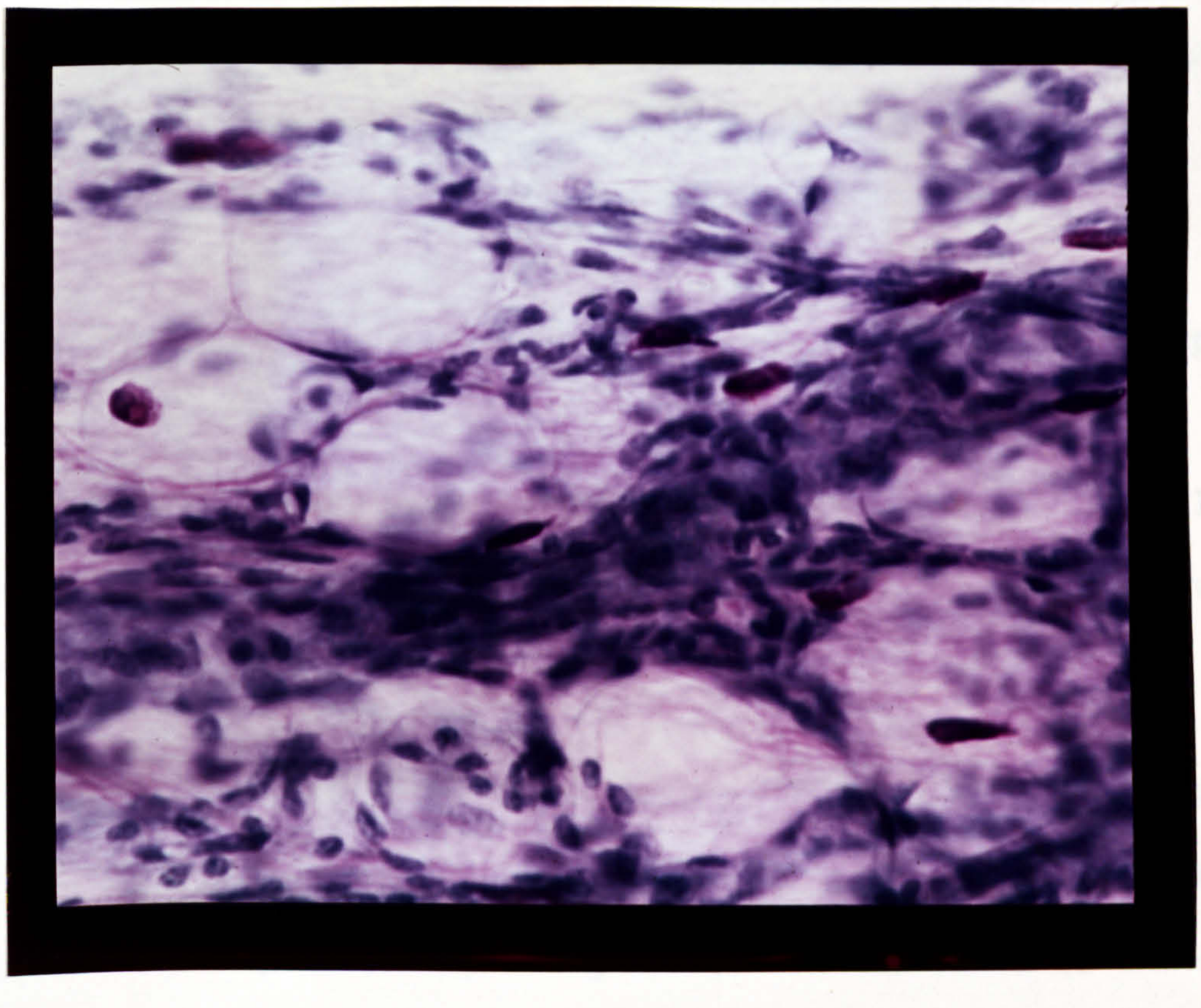


Figure 31. Mast cells in guinea pig subcutaneous connective tissue.

Typical irregularly shaped mast cells were observed in spreads of guinea pig subcutaneous connective tissue. The tissue specimens were spread onto microscope slides, fixed in absolute alcohol, and stained with 1% Toluidine Blue (x 600).

In this particular experiment, four groups of 5 guinea pigs were topically challenged with either 0.9% saline (controls), or standard doses of ovalbumin, histamine, or compound 48/80. The cell counts obtained are shown together with the conjunctival responses (mean ± s.e.m.) and number of cells counted, for each treatment group.

The saline control smears contained predominantly mucosal epithelial cells, and a few lymphocytes. Both of these cell types were present on all of the smears examined. The control smears also contained a small number of neutrophils (3.2%), indicating that these cells are normally present in the conjunctiva. No eosinophils were observed on any control smears.

In contrast, the 24 hour smears taken from antigen, histamine, and compound 48/80 challenged guinea pig conjunctivae all showed considerable numbers of neutrophils, comprising 19.4, 8.8, and 12.3% of the cells present respectively. These neutrophil cell counts were significantly greater than controls in all three cases ($p<0.01$). A substantial eosinophilia was only observed on those smears taken from antigen challenged animals (5.4%). A smaller number of eosinophils were present on smears from compound 48/80 challenged guinea pigs (1.1%), while this cell type was rare following histamine challenge (0.1%).

It was therefore concluded that substantial numbers of neutrophils infiltrate into the conjunctiva as a result of topical challenge with antigen, histamine, or compound 48/80. In contrast, large numbers of eosinophils were only found following antigen challenge. Smaller numbers of eosinophils were present with compound 48/80, whereas none were usually observed in those animals challenged with histamine.

Table 23. Conjunctival smear counts.

CHALLENGE	n	EYE REACTION	CELLS COUNTED	% EOSINOPHILS	% NEUTROPHILS
SALINE	5	0	301 ± 68	0	3.2 ± 0.8
OVALBUMIN	5	2.9 ± 0.4	282 ± 47	5.4 ± 1.1*	19.4 ± 3.9*
HISTAMINE	5	3.2 ± 0.1	304 ± 30	0.1 ± 0.1	8.8 ± 3.4*
COMPOUND 48/80	5	1.8 ± 0.3	225 ± 35	1.1 ± 0.4	12.3 ± 1.8*

The conjunctival smears were taken 24 hours after topical challenge with saline, ovalbumin, histamine, or compound 48/80. The conjunctival responses (visually scored) are expressed as group mean ± s.e.m. The smears were alcohol fixed, stained with Hansel stain, and assessed by differential counts of 200-300 cells in random fields. Neutrophils were present on smears from all treatments including the controls (saline challenge). Eosinophils were only present in significant numbers on the smears taken from ovalbumin challenged guinea pigs.

* significantly different from controls ($p<0.01$).

4.3.2. Paraffin sections:

The conjunctival smear technique allows the sampling and assessment of a limited cell population obtained from the outer conjunctival mucosal surface. It can therefore provide only limited information about the overall distribution of cells infiltrating the conjunctiva. In order to investigate the cellular infiltration more fully, and to study any structural changes in the tissues occurring during or following conjunctival reaction, paraffin sections were prepared and stained with haematoxylin and eosin as described. Eyes were removed from guinea pigs at varying periods after conjunctival challenge as follows: ½, 4, 8, 16, 24, and 48 hours. The presence and distribution of both eosinophils and neutrophils at each of these time intervals was assessed using both the tissue mapping or selective area counting methods.

At ½ hour after challenge, oedema is extensive in the submucosal connective tissue of the conjunctiva, and was responsible for the slight 'breaking up' of some sections. The blood vessels of the submucosal tissue were also found to be markedly dilated at this time. These effects were strictly localized to the submucosal tissue of the conjunctiva itself. No oedema or vascular dilation was observed in the subcutaneous regions of the outer eyelids.

Following all three types of challenge, a characteristic and massive infiltration of neutrophils commenced as early as ½ hour after challenge. Neutrophils first appeared in and around dilated submucosal blood vessels (Figure 32). The number of neutrophils present had increased substantially by 4 and 8 hours after challenge, and was generally maximal at 8 or 16 hours (Figure 33). Infiltration of neutrophils occurred along the

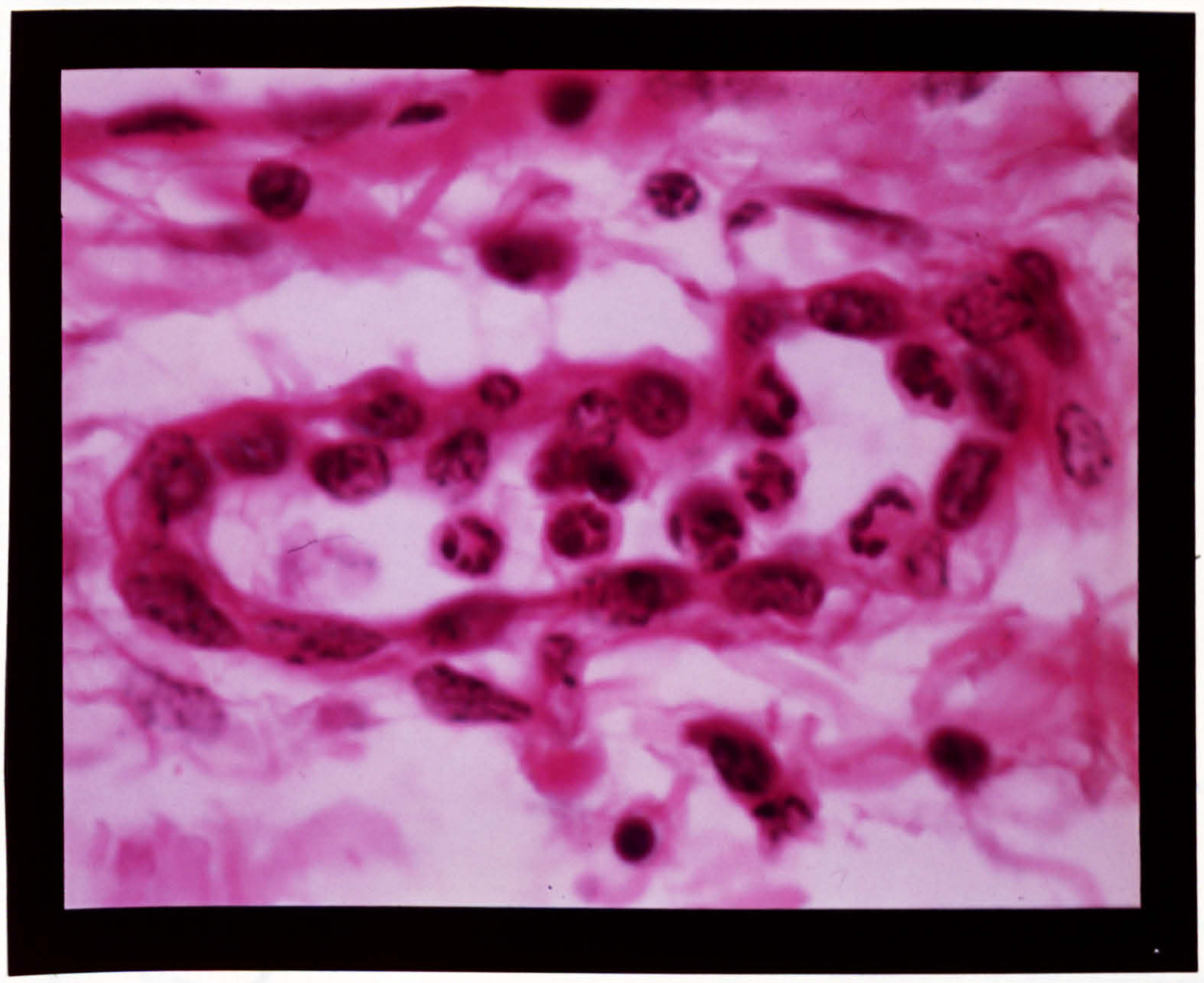


Figure 32. Neutrophils observed in and immediately adjacent to a dilated conjunctival submucosal blood vessel 30 minutes after challenge with ovalbumin. Section fixed in formol-alcohol and stained with haematoxylin and eosin (x 825).

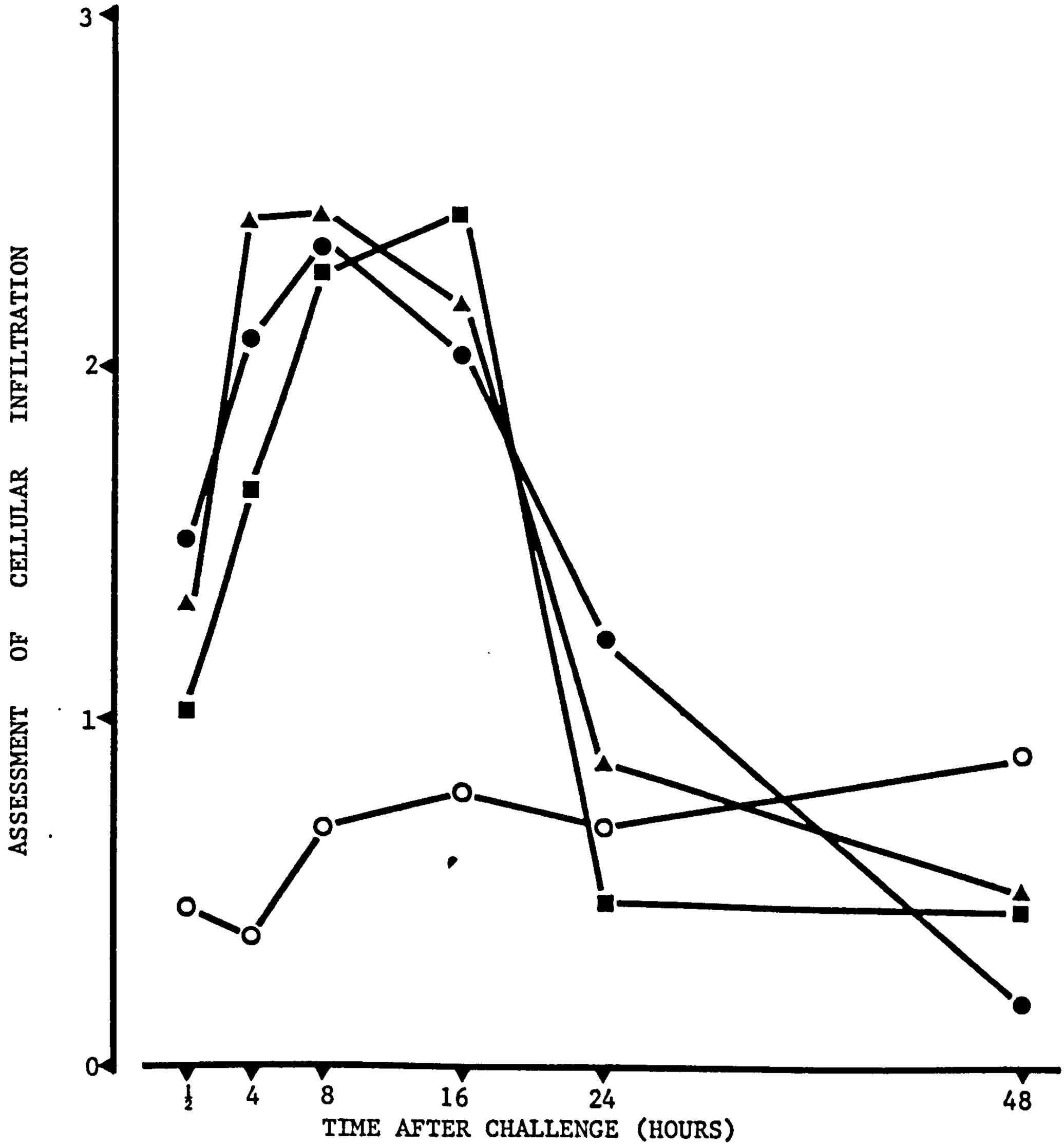


FIGURE 33. The time course of cellular infiltration into the conjunctiva following topical challenge.

Groups of 5-7 guinea pigs were topically challenged with either ovalbumin (500 µg), histamine (250 µg) or compound 48/80 (7·5 mg). Histological analysis by tissue mapping technique was performed on sections of conjunctival tissue obtained from groups sacrificed at each of a series of intervals after challenge. All sections were cut from conjunctival tissue fixed in formol-alcohol, and stained with haematoxylin and eosin. Neutrophils were assessed following ovalbumin (● - ●), histamine (■ - ■) and compound 48/80 challenge (▲ - ▲), and eosinophils following ovalbumin challenge (o - o).

entire length of the conjunctiva in most cases, and at 8 and 16 hours, appreciable numbers of these cells had migrated across the submucosa towards the mucosal basement membrane, and on into the mucosal epithelium itself (Figure 34). A small number of neutrophils appeared to migrate into the outer substantia propria of the cornea, but none were seen throughout the bulk of this tissue. The neutrophil presence had decreased dramatically by 16 and 24 hours on most sections, with few remaining in the conjunctiva by 48 hours after challenge.

The eosinophil response appeared more selective. These cells were only observed in significant numbers following antigen challenge, and were found widely distributed throughout the submucosal tissue, often in the deeper submucosa (Figure 35). Accumulation of eosinophils in the submucosa also took place more slowly. Appreciable numbers were seldom evident before 8 hours after challenge, and usually increased at 16, 24, and 48 hours. Clustering of eosinophils along the mucosal basement membrane, and infiltration into the mucosal epithelium (Figure 36) was also less commonly observed than with the preceeding neutrophil response.

In contrast to the picture following antigen challenge, where an intense neutrophilia preceeded a more generalised eosinophilia, the compound 48/80 induced neutrophilia was followed only by sparse numbers of eosinophils. These were again widely distributed along the length of the submucosa, and only occasionally accumulated close to the mucosal basement membrane. Eosinophils were rare and usually absent following histamine challenge.

Cell counting was performed on preselected areas of the conjunctiva in order to define and quantify more accurately the distribution of neutrophils at each set period following challenge. Neutrophil counts

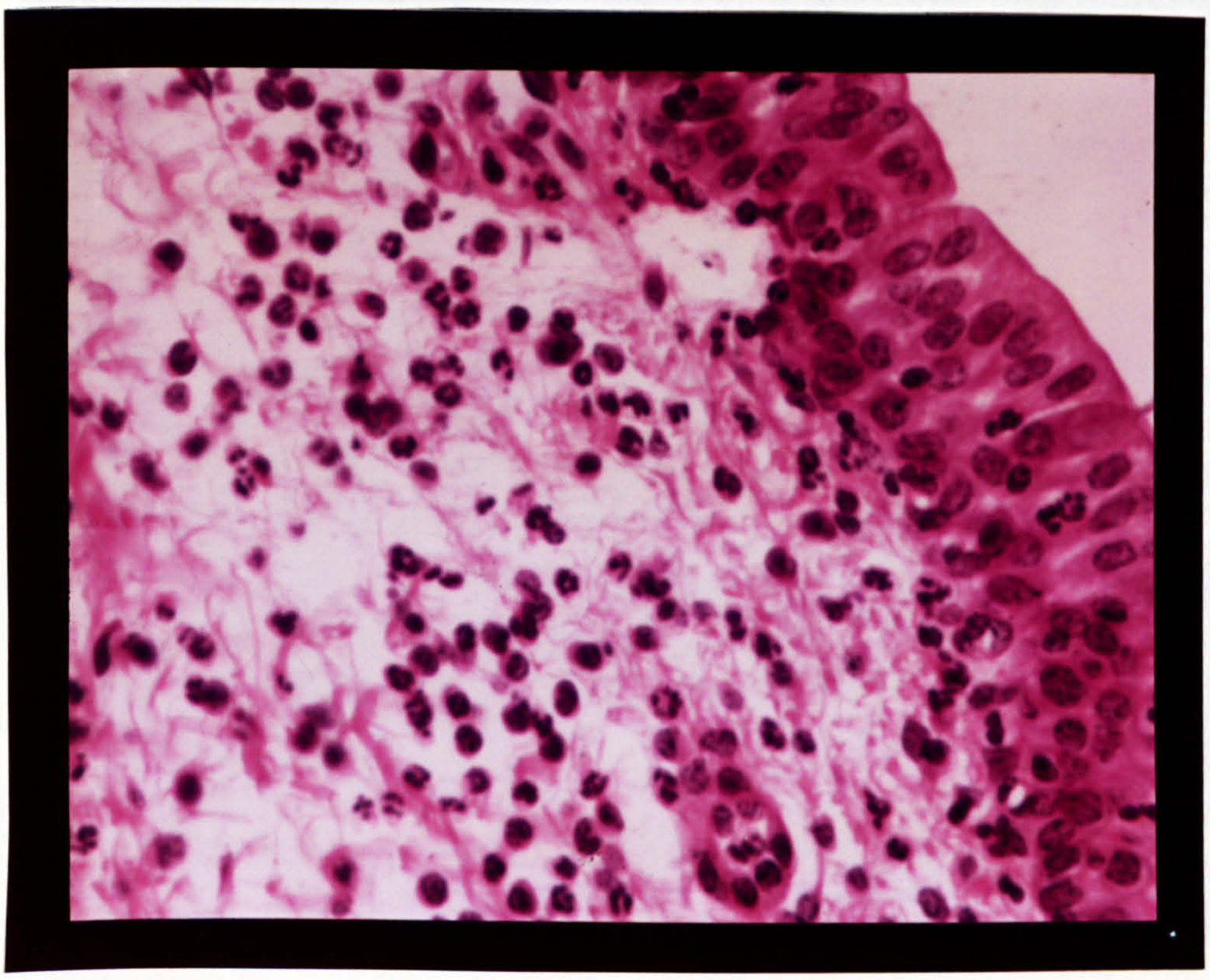


Figure 34. Infiltration of neutrophils into the guinea pig conjunctiva at 8 hours after topical challenge with histamine (250 µg). Neutrophils were numerous at this time, widely distributed throughout the submucosal tissue, and also present in the mucosal epithelial cell layer itself. Section stained with haematoxylin and eosin (x 400).

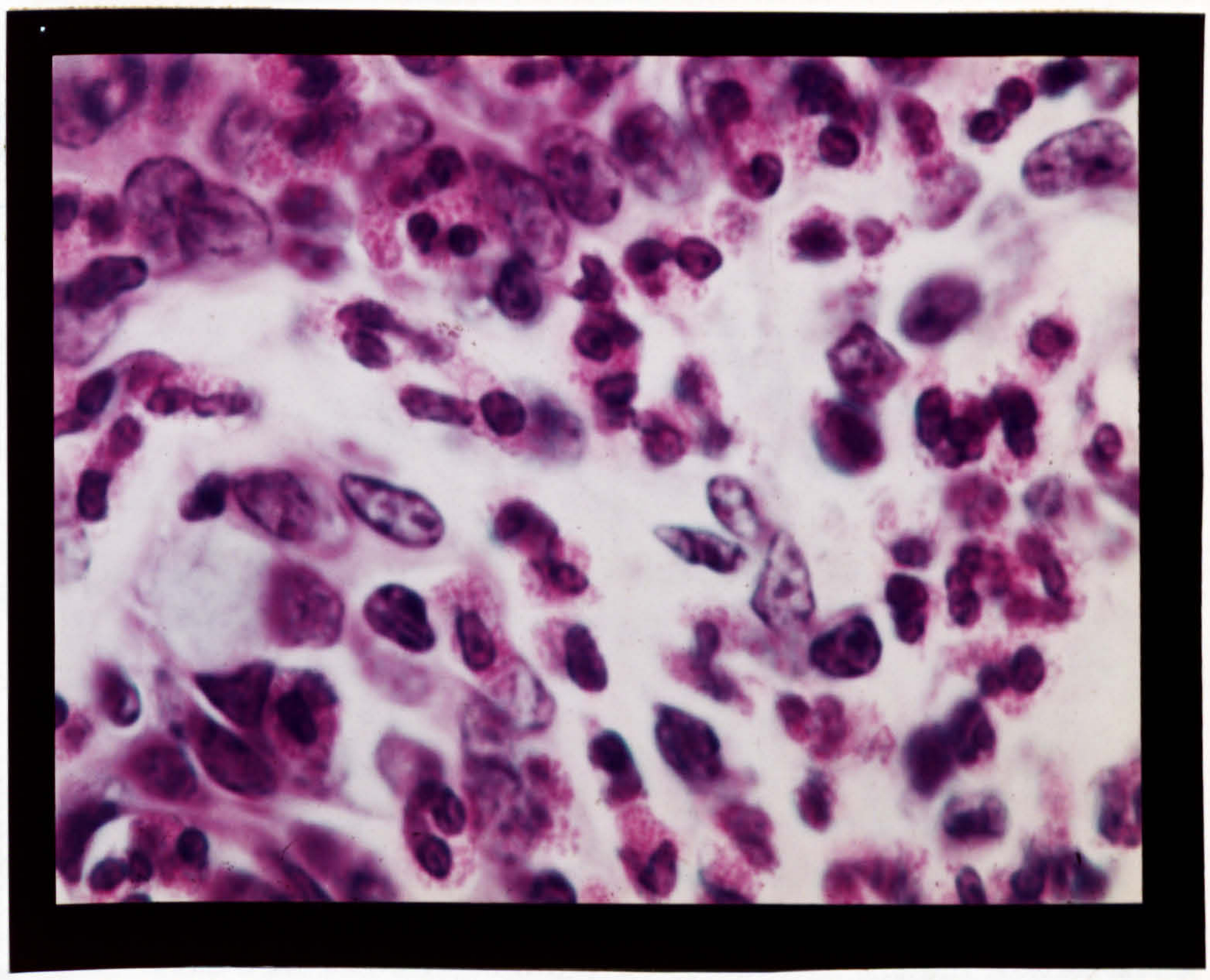


Figure 35. Eosinophils present in the submucosal conjunctival tissue of a guinea pig challenged with antigen 24 hours previously. Section fixed in formol-alcohol and stained with haematoxylin and eosin (x 600).

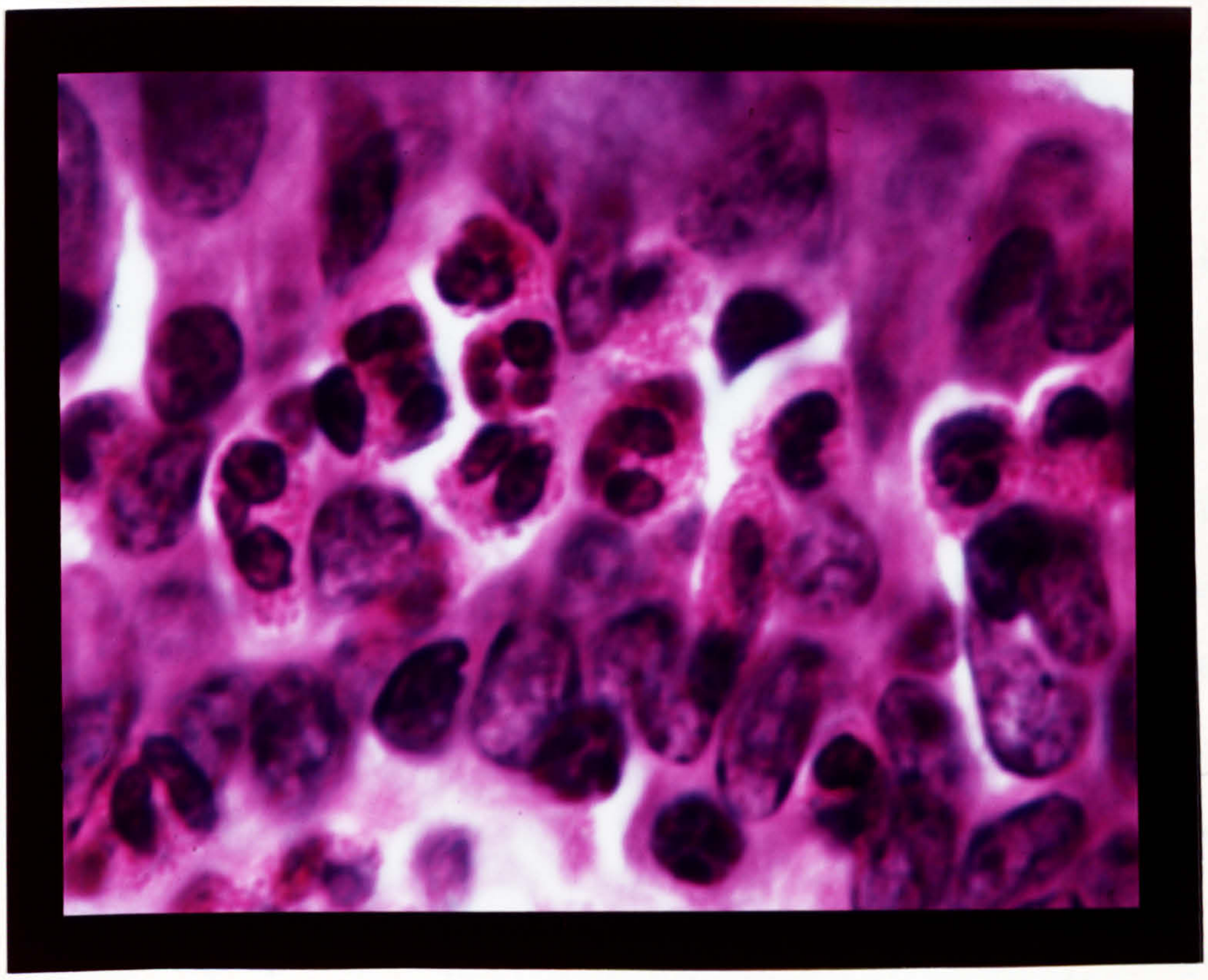


Figure 36. A group of eosinophils observed in the conjunctival mucosal epithelial cell layer of a guinea pig 24 hours after topical challenge with antigen. Section stained with haematoxylin and eosin (x 1500).

in the submucosa were high as early as ½ hour after challenge, and peaked at 4 hours, for each of antigen, histamine, or compound 48/80 challenge (Figure 37). Mucosal neutrophil counts did not peak before 8 or 16 hours, at which time the overall neutrophil infiltration was also maximal for all three types of challenge.

The pattern of cellular infiltration into the conjunctiva seemed therefore to be one of complete contrast between neutrophil and eosinophil participation. Neutrophils arrived quickly following all three types of conjunctival challenge. They then appeared to migrate away from dilated blood vessels into the submucosal tissue, on towards the basement membrane, and frequently into the mucosal epithelium itself. The eosinophilic infiltration which followed only antigen challenge was slower in onset, seldom as dense, and always more widely distributed.

Sections prepared from control guinea pig eyes (unchallenged, saline challenged, or antigen challenged unimmunized animals) showed no significant neutrophil or eosinophil infiltration. In addition, 4 hour neutrophil counts on sections prepared from antigen and histamine challenged complement (C_4) deficient guinea pigs were unchanged from those obtained in the above experiments (Table 24).

4.4. The Effect of Conjunctival Reaction Inhibitors on the Cellular Response.

4.4.1. Triprolidine:

The cell counting technique was used to investigate the effect of triprolidine on the neutrophil infiltration which follows antigen and histamine topical challenge. Guinea pigs were treated with triprolidine (3.0 mg/kg) by intraperitoneal injection 30 minutes prior to challenge.

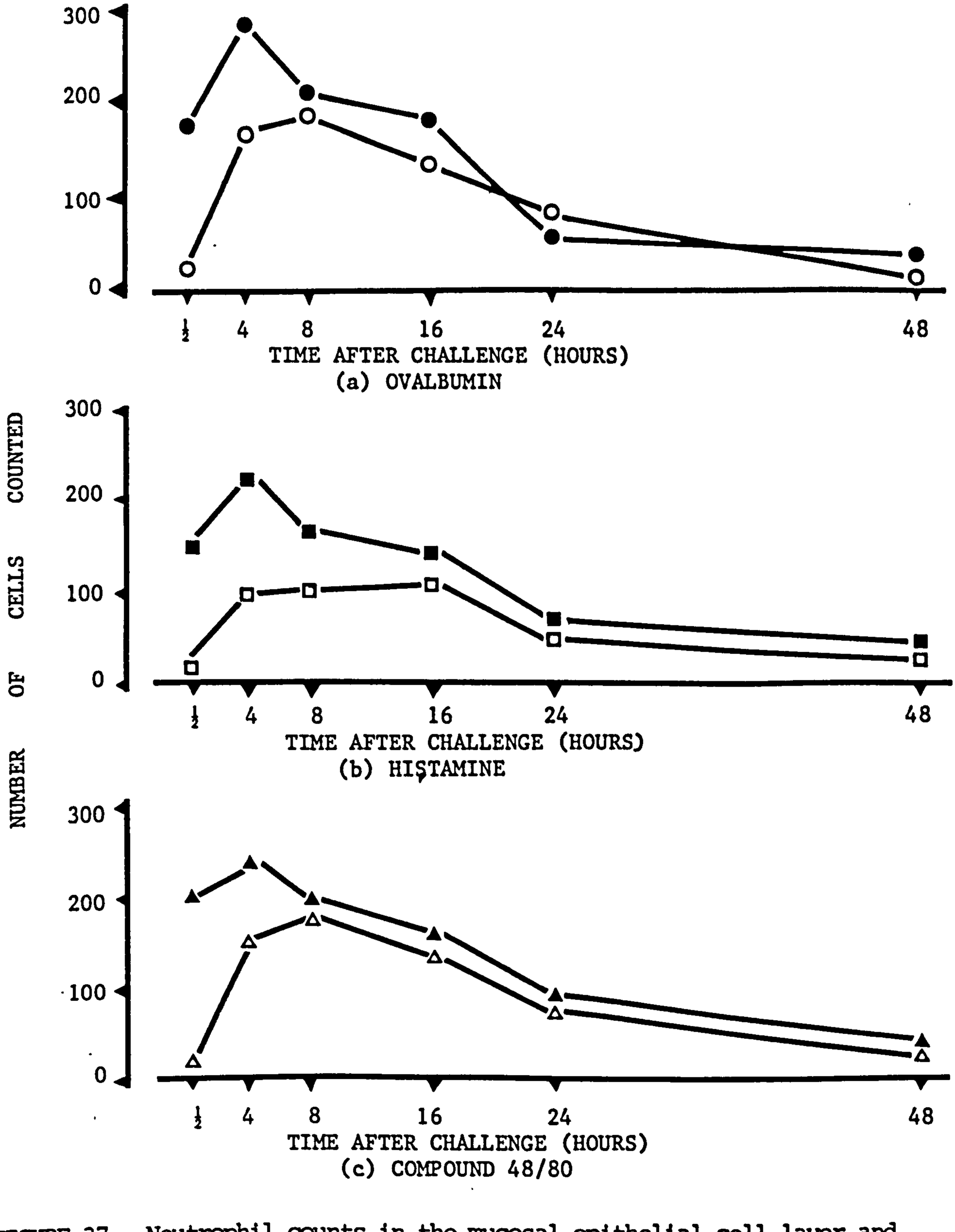


FIGURE 37. Neutrophil counts in the mucosal epithelial cell layer and immediate submucosal tissue following challenge.

Neutrophils were counted in the mucosal epithelial cell layer (open symbols) and immediate submucosal tissue area (closed symbols) following ovalbumin (● / o), histamine (■ / □) and compound 48/80 (▲ / △) challenge. The results are expressed as the mean of > 5 observations for each challenge treatment and time interval.

The triprolidine dose chosen had been previously observed to maximally inhibit the antigen response, and abolish the histamine reaction (Chapter Three).

The submucosal neutrophil counts from sections taken at 4 hours after challenge in triprolidine pretreated guinea pigs decreased to 27% (ovalbumin) and 21% (histamine) of controls (Table 24). The mucosal counts were similarly reduced to 13% (ovalbumin) and 11% (histamine) of expected values. Inhibition of the gross conjunctival reaction with triprolidine had therefore also significantly inhibited the neutrophil cellular response ($p<0.01$) which followed each type of challenge.

4.4.2. Anti-Allergic Agents:

Paraffin sections prepared from the eyes of guinea pigs challenged with antigen in the presence of DSCG and doxantrazole (both 2.5 mg doses) were studied using the tissue mapping technique. In what was only a strictly limited study with a few animals, the results were not as clear cut as those obtained with triprolidine. This was probably due to the less effective and frequently only partial nature of the gross response inhibition observed with DSCG and doxantrazole. Nevertheless, the overall picture on sections examined at both 4 and 24 hours after challenge indicated that when marked inhibition of the antigen response occurred with these drugs, a reduction in the subsequent cellular infiltration also resulted. In those guinea pigs where the gross response had only been partially inhibited, little apparent difference was noted in either the density or distribution of the subsequent neutrophilic or eosinophilic infiltrations.

Table 24. Mucosal and sub-mucosal neutrophil counts in normal, complement (C4) deficient, and triprolidine treated guinea pigs at four hours after challenge with ovalbumin and histamine.

Protocol			4 hour Neutrophil cell count (mean).	
Challenge	n	Treatment	Mucosal epithelium	Sub-mucosal tissue
Saline	12	unimmunized	3.8	3.9
Ovalbumin	5	unimmunized	14.0	12.0
Ovalbumin	16	immunized	170.1	289.1
Histamine	8	immunized	99.4	221.7
Ovalbumin	4	immunized	146.8	472.0
Histamine	4	C4 def.	101.8	296.8
Ovalbumin	5	immunized	22.6	77.6
Histamine	5	Triprolidine	11.0	46.6

Neutrophil cell counting in predefined mucosal and sub-mucosal tissue areas was performed as described in Materials and Methods section. The cell counts for the ovalbumin immunized, triprolidine (3.0 mg/kg) treated guinea pig group were significantly different from control immunized values (Student's t-test: $p<0.001$), while those for the complement (C4) deficient group were not.

D I S C U S S I O N

Perhaps surprisingly, there have been few previous reports describing the distribution of mast cells in the conjunctival tissue of either man or experimental animals (see Riley, 1959; Selye, 1965). In the present study, these cells were observed predominantly in the sub-mucosal tissue of the guinea pig conjunctiva in the region of the basement membrane. Although previous reports have shown that these cells are common in certain areas of the skin of this species, few were present in the outer skin of the eyelid.

In both human and guinea pig lung tissue, mast cells are numerous in the mucosa of the trachea, and are frequently evident in the sub-mucosal tissue close to the pulmonary blood vessels of the larger bronchi (Mota and Vugman, 1956a; Salvato, 1961; Selye, 1965; Brinkman, 1968). They are also situated immediately below the epithelial basement membrane of the bronchial mucosa in a similar type of tissue area to those observed in the guinea pig conjunctiva during the present study (Staub, 1955; Richardson et al., 1973). Clearly, mast cells are widely distributed throughout the body, and are common in those mucous membrane tissues exposed to the external environment, being also found in the submucosal tissue of the intestinal tract (Norris et al., 1963; Thompson et al., 1964) and nasal passage (Boreus, 1960; Ali, 1964).

In the present investigation, a characteristic neutrophilic infiltration was found to follow topical conjunctival challenge with antigen, histamine, or compound 48/80. Arriving within 30 minutes of challenge, neutrophils were most evident in the mucosal and submucosal tissue at between 8 and 16 hours. In contrast, eosinophils were only

observed in significant numbers following antigen challenge, over an extended period between 8 and 48 hours after conjunctival reaction (Figure 33). A smaller number of eosinophils were present on sections prepared from compound 48/80 challenged eyes, while few or none resulted from histamine challenge.

The importance of the local neutrophilic and eosinophilic infiltrations commonly observed following anaphylactic reactions in tissues such as the conjunctiva remains largely obscured by our inadequate knowledge of the mechanisms which govern the movement and function of these cell types. Three areas of considerable interest in recent years have therefore been (i) how is the bone marrow stimulated to increase stem cell production, maturation, and release of reticulo-endothelial cells, (ii) when and why do local stimuli direct cells to leave the blood and infiltrate specific tissue areas, and (iii) what is the function of these cells once they are present in inflammatory or anaphylactic reaction sites?

Neutrophils are known to be highly active phagocytic cells for either bacteria or inert foreign particles, a process which is frequently accompanied by degranulation (Wilson, Wiley, and Bruno, 1957; Hirsch and Cohn, 1960; Sbarra et al., 1961). The lysosomal granules of neutrophils have been shown to contain phosphatases, hydrolytic enzymes, and a variety of other bactericidal substances (Cohn and Hirsch, 1960; Horn and Spicer, 1964; Horn, Spicer, and Wetzel, 1964; Klebanoff, 1971). They have also been reported to actively phagocytose and degrade antigen-antibody complexes (Cochrane, Weigle, and Dixon, 1959; Sorkin and Boyden, 1959; Hensen, 1971), and frequently appear to monitor the persistence and severity of an inflammatory reaction, in addition to serving merely as a phagocyte.

The significant contribution of the neutrophil to the protection of the body from bacterial invaders, the phagocytosis of immune complexes, and the release of active inflammatory substances, has therefore been clearly established. The role of the eosinophil has attracted rather greater speculation.

Archer (1960, 1963) demonstrated that the injection of histamine into horse skin attracts eosinophils, and suggested that the function of these cells might be the inactivation of anaphylactic mediators such as histamine, 5-hydroxytryptamine, and bradykinin. Eosinophils have also been observed in the skin of human atopic subjects following histamine application (Eidinger, Wilkinson, and Rose, 1964; Atkins, Green, and Zweiman, 1973). In contrast, none arise from the intradermal injection (Parish and Coombs, 1968) or topical conjunctival instillation (this study) of histamine in guinea pigs.

A number of reports in different species have shown that eosinophils are able to selectively phagocytose antigen (Roberts, 1966), antigen-antibody complexes (Archer and Hirsch, 1963b; Sabesin, 1963; Litt, 1964a; Parish, 1970a), bacteria (Cline, Hanifin, and Lehrer, 1968), foreign red blood cells in the presence of immune serum (Archer and Bosworth, 1961), and mast cell granules following antigen or compound 48/80 induced exocytosis (Welsh and Greer, 1959; Mann, 1969). In man, eosinophils from ragweed-sensitive patients also possess ragweed antigen E binding activity (Hubscher and Eisen, 1971), and take up IgE-ragweed antigen immune complexes (Ishikawa, Wicherk, and Arbesman, 1974; Fujita et al., 1975).

Eosinophil lysosomal granules, the contents of which have been detected in phagocytic vacuoles surrounding ingested material, are known to possess a high peroxidase core content. Other hydrolytic

enzymes present include acid phosphatases, aryl sulphatase, β-glucuronidase and ribonuclease (Archer and Hirsh, 1963a, 1963b; Miller, De Harven, and Palade, 1966; Bainton and Farquhar, 1968a, 1968b; Gessner, Shelton, and Himmelhoch, 1972; Gleich, Loegering, and Maldonado, 1973). Eosinophil granules also contain histaminase and phospholipase D (Goetzl, 1976), an SRS-A destructive activity attributed to aryl sulphatase (Orange, Murphy, and Austen, 1974; Wasserman, Goetzl, and Austen, 1975), and anti-histamine properties demonstrable on smooth muscle (Vercauteren and Peeters, 1952). In addition, Hubscher (1975a, 1975b) has described an eosinophil derived mast cell mediator release inhibiting factor, which possibly acts by increasing cAMP levels in the mast cell. This factor is a mixture of acidic lipids resembling PGE_1 and PGE_2 in biological activity, and its release is blocked by indomethacin (the important effects of PG's on mast cell cAMP levels and mediator release are more fully discussed in Chapter Three).

The stimulus for the specific directional locomotion of eosinophils and neutrophils into reactive tissue sites such as the conjunctiva has been widely attributed to the local biosynthesis of specific chemotactic factors. These are either released, or directly activated, during anaphylactic and inflammatory reactions. The whole subject of leucocyte chemotaxis and its significant role in the inflammatory response has been recently reviewed by Wilkinson (1974). Boyden (1962), using an *in vitro* chamber technique, was the first to show that antigen-antibody complexes incubated in the presence of immune serum are chemotactic for polymorphonuclear leucocytes. Histamine, 5-HT, and SRS-A were not chemotactic in the same system (Kay and Austen, 1971). Further studies (Keller and Sorkin, 1965; Kay, Stechschulte, and Austen, 1971) indicated that the chemotactic activity observed with immune complexes is serum

complement dependent (immune complexes incubated in the presence of heated immune serum appear chemotactically inactive *in vitro*), rather than being a direct effect of the complexes alone.

At least three components of complement have been shown to possess chemotactic activity *in vitro* for eosinophils and neutrophils. These are C3a, a split product of complement activation also possessing anaphylatoxin activity, and having a molecular weight of 7-10,000 (Bokisch, Muller-Eberhard, and Cochrane, 1969), C5a, also a split product with a molecular weight of approximately 15,000 (Snyderman et al., 1969; Kay, Stechschulte, and Austen, 1971; Kay, Shin, and Austen, 1973), and $\overline{C567}$, a 200,000 molecular weight complex arising from C1-C7 activation (Ward, Cochrane, and Muller-Eberhard, 1965, 1966).

It is now widely recognised that lymphocytes are also capable of releasing substances which possess selective chemotactic activity for either eosinophils or neutrophils. These substances belong to a group of soluble factors, termed lymphokines, which are released when lymphocytes are incubated *in vitro* with antigen, antigen-antibody complexes, or mitogens such as phytohaemagglutinin (Cohen and Ward, 1971; Pick and Turk, 1972; Torisu et al., 1973). However, it is more likely that lymphokines are responsible for the increases in blood eosinophil counts observed during parasitic infections, rather than the specific local infiltrations which follow immediate hypersensitivity reactions.

An active contribution of complement-derived chemotactic factors to the anaphylactic cellular response also appears doubtful. The complement (C4) deficient guinea pigs tested in the present study showed normal gross reactions and subsequent cellular responses, to antigen and histamine conjunctival challenge. Although alternative pathway complement

activation, and direct C3 or C5 enzyme cleavage are both possible in C4 deficient animals, decomplementation with cobra venom factor is also reported to leave eosinophil and neutrophil accumulation at IgG_1 guinea pig passive cutaneous anaphylaxis sites unaffected (Cochrane, Muller-Eberhard, and Aikin, 1970; Ellman et al., 1971; Kay and Austen, 1972).

The precise mechanism of eosinophil chemotaxis following immediate hypersensitivity reactions remains to be determined, and is probably the result of multiple chemotactic factor activities. Nevertheless, a number of recent investigations have shown that a factor selectively chemotactic for eosinophils is released from a variety of tissues including human blood basophils, human and guinea pig chopped lung, and rat peritoneal mast cells, during anaphylactic reactions (Kay and Austen, 1971; Kay, Stechschulte, and Austen, 1971; Parish, 1972c; Lewis et al., 1975). This substance, a low molecular weight peptide, exists preformed in the tissues, and has been designated eosinophil chemotactic factor of anaphylaxis (ECF-A). Its release is antigen dose dependent, and is modulated by drugs affecting intracellular cAMP levels in the same way as that of histamine or SRS-A (Wasserman et al., 1973; Wasserman, Goetzl, and Austen, 1974). The structure of ECF-A has been established as a mixture of two tetrapeptides of the amino acid sequences Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (Goetzl and Austen, 1975, 1976).

The infiltration of eosinophils into the conjunctiva of the guinea pig following topical challenge with antigen or compound 48/80 may therefore be attributed at least in part to the release of ECF-A from target cells. However, if lymphokines and complement derived chemotactic factors are not involved in the conjunctival cellular response, the stimulus for the initial (15-30 minutes) and sustained (4-16 hours)

neutrophilic infiltration remains to be identified.

Parish (1972a, 1972b), during a series of histological studies on IgG_1 mediated guinea pig PCA reaction sites, has also described an early neutrophilia and a subsequent eosinophilia of slower onset. These findings bear a close resemblance to the pattern in the guinea pig conjunctiva. He suggested that at the height of an anaphylactic reaction, during the phase of capillary dilation and increased permeability, cells may migrate into anaphylactic tissues sites in the relative proportions in which they are present in the bloodstream. The infiltration of additional cells might then be stimulated by the release of specific chemotactic factors either during the initial tissue reaction (e.g. ECF-A), or as a result of phagocytosis of foreign material and/or immune complexes by the first inflammatory cells (i.e. neutrophils) to arrive.

There is considerable evidence to support the above hypothesis. Not only are neutrophils among the first cells to arrive at the inflammatory tissue sites characteristic of a number of clinical disorders, but they have also been shown to release a number of agents chemotactic for neutrophils and eosinophils. These include PGE_1 (Higgs, McCall, and Youlten, 1975), thromboxane B_2 (Boot, Dawson, and Kitchen, 1976), the arachidonic acid lipoxygenase metabolites HPETE and HETE (Vane, 1976; Turner, Tainer, and Lynn, 1976), and a neutrophil derived eosinophil chemotactic factor (Czarnetzki, Konig, and Lichtenstein, 1976; Konig, Czarenetzki, and Lichtenstein, 1976). In the present investigation conjunctival challenge with histamine produced a severe conjunctival response, followed by a substantial neutrophil infiltration as shown in Figures 32 and 34. Both the conjunctival oedema and erythema, and the resultant tissue neutrophilia, were abolished by pretreatment with an

anti-histamine. Clearly, the two phenomena are closely associated.

In conclusion, if the infiltration of eosinophils into the conjunctiva observed between 8 and 48 hours after challenge is largely due to release of ECF-A from mast cells, it should remain unaffected by pretreatment of guinea pigs with doses of H_1 receptor antagonists such as triprolidine. It should also be possible to mimic the response with synthetic chemotactic tetrapeptides. On the other hand, if the selective chemotaxis of eosinophils into conjunctival tissue is dependent in any way on the preceeding neutrophil infiltration, then triprolidine should actively inhibit the arrival of both cell types. This remains to be investigated. Furthermore, if the biosynthesis of thromboxanes or prostaglandins at the local tissue site is important, then although the prostaglandin synthetase inhibitor indomethacin was inactive against the gross conjunctival reaction, it too might be expected to inhibit the subsequent cellular response.

GENERAL DISCUSSION

The present study was designed to investigate immediate conjunctival hypersensitivity in the guinea pig as a potentially useful animal model for allergic conjunctivitis in man. In this respect, four important criteria on which any conclusions must be based are:

(1) does the experimentally induced guinea pig conjunctival response closely resemble the symptoms characteristic of human allergic conjunctivitis?

(2) are the immunological, pharmacological, and pathological mechanisms of both human and guinea pig responses similar in nature?

(3) is the guinea pig experimental model selectively sensitive to the action of drugs which possess proven clinical efficacy, such as anti-histamines or disodium cromoglycate?

(4) is the guinea pig conjunctival model appropriate for the routine testing of novel anti-allergic compounds?

The results described in the text have both confirmed and extended the initial observations of Feinberg and Chopra (1966). Topical instillation of either serum or protein antigens onto the conjunctival tissue of correspondingly immunized guinea pigs consistently resulted in evidence of local irritation and lacrimation. This was followed by a rapidly developing erythematous and oedematous response which extended throughout the bulbar and palpebral conjunctiva. The guinea pig conjunctival response therefore bore a close resemblance in this respect to the clinical conditions observed in man.

It should be remembered that the guinea pig immediate conjunctival hypersensitivity reaction is an acute response to a single dose antigen challenge. In direct contrast, human allergic conjunctivitis is frequently a chronic response to a lower level of continual antigen exposure, which may continue over a period of hours or days. Criticism regarding duration of response may be equally applied to a number of other widely used experimental models of allergy. Most of these models are single dose acute reaction tests, including passively sensitized human chopped lung or rat peritoneal mast cells *in vitro*, and antigen induced bronchoconstriction or passive cutaneous anaphylaxis *in vivo*. However, in the case of guinea pig conjunctival anaphylaxis, the data obtained in Chapter One of this thesis indicated that with a modified protocol, a low level response could be developed and maintained for a prolonged period. This might more closely resemble the human clinical situation.

A whole range of anti-histamines such as promethazine, mepyramine, and triprolidine, given either systemically or topically, have been extensively employed in the control of human allergic rhinitis and conjunctivitis (Crompton, 1973). The significant protection afforded by these drugs serves to re-emphasise the important contribution of histamine to the human allergic conjunctival response. The potent inhibitory activity of triprolidine against conjunctival anaphylaxis in the guinea pig strongly implies that histamine also plays an important role in this species. Indeed, of all those pharmacological agents tested in the guinea pig conjunctiva, only histamine was observed to produce the intense conjunctival erythema and oedema characteristic of the antigen reaction. In direct contrast, guinea pig conjunctival

sensitivity to 5-HT was poor, even at dose levels considerably higher than those at which histamine was active. Moreover, the inhibitory effects of B.W. 501C67 and methysergide on the guinea pig conjunctival reaction were only marginally significant, and non-specific in nature. Both of these 5-HT antagonists also inhibited the conjunctival response to histamine.

No evidence was obtained to directly implicate the involvement of prostaglandins in the guinea pig conjunctival reaction. Intra-conjunctival injections of PGE_1, PGE_2, and $PGF_2\alpha$ all proved inactive in the guinea pig at dose levels which are known to cause erythema in the skin of guinea pigs and rats (Crunkhorn and Willis, 1969; Williams and Morley, 1973). Pretreatment of test animals with indomethacin, a prostaglandin synthetase inhibitor, also had little effect on the severity of the antigen conjunctival response. The potentiating effect of PGE_1 on the conjunctival responses to antigen and histamine served only to show that endogenous biosynthesis of PG's, if present, might certainly affect the strength of the response.

From these results it seems certain that histamine makes a significant contribution to guinea pig conjunctival anaphylaxis, while a major role for 5-HT and PG's appears doubtful. Future studies should include investigation of conjunctival sensitivity to other pharmacological mediators which are either established or potential contributors to allergic and inflammatory reactions. These include SRS-A, bradykinin, anaphylatoxins (C3a and C5a), and rabbit aorta contracting substance (PGG_2, PGH_2 and thromboxane A_2).

The severity of the guinea pig conjunctival response to antigen

was significantly reduced in the presence of the anti-allergic agents DSCG and doxantrazole. Non-specific anti-histamine activity appeared to contribute to the inhibitory effect of doxantrazole at the highest intravenous and topical doses employed. In contrast, DSCG possessed no anti-histamine activity at its effective anti-allergic doses, confirming a previous report by Cox et al., (1970). The topical doses of these drugs required to inhibit guinea pig conjunctival anaphylaxis were high in both cases. However, in the case of anti-allergic drugs in general, and DSCG in particular, the challenge system, target tissue, and homocytotropic antibody involved, have all been shown to strongly influence the doses required for *in vivo* anti-allergic activity. Furthermore, when a drug is applied topically to the guinea pig conjunctiva, the effective dose in terms of tissue levels attained is hard to estimate, and may only be a fraction of the total dose instilled. A large proportion of the drug dose is probably lost through a combination of scratching, blinking, lachrymation, and drainage from the conjunctival sac. Nevertheless the doses of DSCG required to inhibit guinea pig conjunctival anaphylaxis are broadly comparable to those chosen and effective in human clinical use, recently quoted as 1 drop of a 4% ophthalmic solution 4 times daily (Greenbaum et al., 1977; Easty, 1977).

The potent inhibitory activity of salbutamol against the antigen conjunctival response is in agreement with previous reports that $beta_2$-adrenoceptor stimulants are effective inhibitors of immediate hypersensitivity reactions. This activity has been widely attributed to the prevention of mediator release through adenyl cyclase stimulation, and a resultant increase in mast cell cAMP levels. However, the fact

that salbutamol also effectively inhibited the conjunctival response to histamine at similar dose levels, indicated that prevention of mediator release is not the complete explanation. Two other recent reports have also demonstrated the inhibitory activity of beta$_2$-adrenoceptor agonists against the cutaneous response to histamine in man (Jorde and Schata, 1976), and the exudation of dye which follows intraperitoneal injection of histamine in rats (Smith, Spicer, and Ross, 1977). This is presumably a beta-receptor specific direct effect on the local vasculature.

In man, IgE is now recognised as the major contributory homocytotropic antibody to anaphylactic reactions. In the guinea pig, the situation is less clear, with the possibility of two distinct antibody classes being involved. As in man, guinea pig IgE is a long term sensitizing and heat labile homocytotropic antibody. IgG$_1$ (gamma-1), on the other hand, is a heat stable antibody, and its two subclasses show short (4-8 hours) and medium (2-4 days) term sensitizing activities in passive transfer experiments. A major problem in the guinea pig is therefore the determination of the relative contribution of each of these antibodies to a given anaphylactic response.

As has been the case in several previous studies (Mota and Perini, 1970; Parish, 1970c; Margni and Hajos, 1973a), the PCA data obtained in the present investigation was inconclusive, and the problem remained largely unresolved. IgG$_1$ antibodies possessing short and medium term sensitizing activities were demonstrated in all sera tested by PCA. In those guinea pigs topically challenged regularly over an extended period, short and medium term PCA, and serum haemagglutinating activities, increased substantially. A highly significant correlation was found

between the respective 4 hour PCA and HA activities in those sera tested, indicating that the same IgG_1 antibody populations may be responsible for both immunological properties. Long term (8 day) PCA activities were low or absent in all of the sera taken from guinea pigs immunized with ovalbumin. Haemolytic activity was also absent in all sera tested, confirming the previous observation that immunization with low doses of protein antigens in saline does not induce the formation of haemolytic (IgG_2 and IgM) antibodies in guinea pigs (Benacerraf, Ovary, Bloch, and Franklin, 1963).

The high serum IgG_1 and low IgE serum antibody activities recorded in the PCA studies cannot be taken as precluding the involvement of IgE in the conjunctival response at mast cell level. For example, tissue mast cell bound antibodies are not detected in the PCA test, and other studies have shown that IgE blood levels in guinea pigs may peak as early as 11-13 days after immunization (Margni and Hajos, 1973a; Taylor and Roitt, 1973), i.e. before the serum samples were obtained in the present investigation. In addition, the short and medium term PCA activities detected did not correlate with the severity of individual conjunctival responses in any of the experiments where this possibility was considered. Whereas the short and medium term serum PCA activities increased progressively with regular topical challenge, the strength of the conjunctival responses remained constant. Serum antibody activities in the PCA test, therefore, provide at best only a tentative guide to the class of mast cell bound homocytotropic antibody upon which conjunctival sensitivity to antigen challenge finally depends. On the basis of the results obtained in the present investigation, both IgG_1 and IgE antibodies may contribute to the guinea pig antigen conjunctival

response when the animals are immunized using the protocol employed here.

A characteristic cellular infiltration into the conjunctival tissue of guinea pigs was found to follow each of the three types of conjunctival reaction investigated. Neutrophils were observed in the submucosal conjunctival tissue within 30 minutes of challenge with ovalbumin, histamine, or compound 48/80. This rapidly developing neutrophilia was maximal at either 8 or 16 hours after challenge in all three cases, and receded over the subsequent 12 to 36 hours. In direct contrast, infiltration of eosinophils was only substantial following challenge with antigen over the period between 16 and 48 hours. Eosinophils were observed in smaller numbers as a result of challenge with compound 48/80, but were not generally present following a histamine response. In man, the presence of eosinophils in conjunctival scrapings is frequently taken as indicating the occurrence of an allergic reaction in the conjunctiva, and the appearance of these cells is regarded as a valuable diagnostic tool.

From the results described in the text, it is apparent that the guinea pig conjunctiva possesses certain advantages over other tissues in the investigation of the type, pattern, and mechanism of cellular infiltrations which follow hypersensitivity reactions. Firstly, the nature of the cellular response to a given stimulus may be studied either by the preparation of sequential conjunctival smears, or by paraffin sections obtained at predetermined intervals after challenge. Secondly, antigens, anaphylactic mediators, or other inflammatory substances may be applied topically to the conjunctiva, removing the additional complication of local tissue trauma due to an injection. The guinea pig conjunctiva has already been used by other workers to

study the nature of delayed (Type IV) hypersensitivity reactions in addition to the immediate anaphylactic response (Feinberg, unpub. obs.; Dwyer and Darougar, 1971), and should prove ideal for the investigation of the chemotactic activity of substances such as ECF-A, C3a and C5a, and prostaglandins *in vivo*.

CONCLUSION:

The conjunctival anaphylactic response in the guinea pig certainly appears worthy of further consideration as an experimental *in vivo* model of human allergic conjunctivitis. It involves the correct target tissue, the naturally occurring route of antigen challenge, and provides the option of systemic or local drug administration. The conjunctival response closely resembles that which occurs in man in terms of both gross and cellular components, is easily quantifiable, and selectively inhibited by the same types of drug. Immediate hypersensitivity in the guinea pig conjunctiva therefore appears to satisfy each of the four criteria previously considered relevant in the assessment of this *in vivo* allergy model.

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